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THE UNIVERSITY OF ALBERTA

Cyclosporin Production by *Tolypocladium niveum* strains

by

Charles Edward Isaac

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA
FALL, 1988

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Date Sept. 30. 1988

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Cyclosporin Production by *Tolypocladium niveum* submitted by Charles E. Isaac in partial fulfillment of the requirements for the degree of Master of Science.

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Date: June 28, 1988

Abstract

In view of low productivity of the parent strain of *Tolypocladium inflatum* NRRL 8044, deposited by the Sandoz group, ten isolates of *T.niveum* (=*inflatum*) were examined as an alternate source of cyclosporins. Many *T. niveum* isolates, not previously described as cyclosporin producers, gave higher cyclosporin levels than the parent NRRL strain. Values of 100-200 mg/L were initially observed but production was erratic. A systematic examination of factors affecting peptide production was carried out to standardize and optimize reproducible cyclosporin levels. This included biological aspects such as inoculum standardization and medium development as well as chemical aspects such as extraction procedures and rigorously identifying cyclosporins by mass spectrometry. The best method of quantification was High Performance Liquid Chromatographic analysis and a bioassay with *Aspergillus niger* qualitatively showed biological activity.

The optimum production culture inoculum was found to be a 10% unwashed inoculum grown in a preculture inoculated with 10^8 spores (100 mL). Low concentrations of spores resulted in a pelletized morphology while higher concentrations gave filamentous growth and higher cyclosporin levels (200 mg/L). The optimum preculture and production medium was found to be 2% sorbose, 1% vitamin assay casamino acids, 1% KH_2PO_4, and 0.5% KCl autoclaved *in situ*. Supplementation of the medium with constituent amino acids allowed direction of cyclosporin synthesis to particular classes and increased production (>200 mg/L). The addition of ^{14}C-methyl methionine resulted in the production of radiolabelled cyclosporin. Time course studies demonstrated that cyclosporin levels and biomass increase rapidly to day 10 (180 mg/L and 10 g/L respectively) while the medium components were rapidly taken up. Production in three fermenter designs showed cyclosporin could be produced at high levels in both a 1L external loop airlift and a 10L stirred tank fermenter (>100 mg/L).

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TABLE OF CONTENTS

CHAPTER PAGE #

1 LITERATURE REVIEW AND OBJECTIVES.................................... 1
1.1 Literature Review.. 1
1.1.1 Cyclosporin: A Fungal Metabolite.................................. 1
1.1.2 Chemical Structure of Cyclosporin................................ 3
1.1.2.1 Detection of Cyclosporin.................................. 7
1.1.3 Biological Activity.. 8
1.1.4 Directed Synthesis of Cyclosporins................................ 12
1.1.5 RadioisotopeLabelling.. 12
1.1.6 Fermenter Design and Fermenter Production of Cyclosporin. 14
1.1.7 Medium Composition and Growth Conditions................. 17
1.1.8 Nutritional Studies.. 18
1.1.9 Chemical Synthesis of Cyclosporin................................ 19
1.1.10 Cell Free Synthesis of Cyclosporin.............................. 20
1.1.11 Peptide Antibiotics as Secondary Metabolites.................. 21
1.2 Objectives.. 25
1.2.1 Screening of Cyclosporin-Producing Isolates................ 26
1.2.2 Development of Methods.. 26
1.2.3 Medium Development.. 27
1.2.4 Scale Up of Cyclosporin Production.......................... 27
2 METHODS AND MATERIALS.. 28
2.1 Microbiological.. 28
2.2 Growth in Submerged Culture.. 28
2.3 Extraction of Cyclosporins from Culture Homogenates................ 30

CHAPTER PAGE #

2.3.1 Comparison of Extraction Methods.............................. 31

2.4 High Performance Liquid Chromatographic Analysis for Cyclosporins.............................. 31

2.5 Bioassays for Cyclosporin Activity.............................. 32

2.6 Analytical Methods.............................. 37

2.6.1 Production of Radiolabelled Cyclosporin.............................. 37

2.6.2 Liquid Scintillation Counting Methods.............................. 38

2.6.3 Identification of Cyclosporins by Mass Spectroscopy and HPLC Elution.............................. 38

2.7 Fermenter Designs.............................. 39

2.8 Immobilization of *T. niveum*.............................. 40

2.9 Methods of Sorbose Sterilization.............................. 40

2.10 Statistical Analysis.............................. 41

2.11 Chemicals.............................. 41

3 RESULTS.............................. 42

3.1 Initial Comparison of *Tolypocladium* and *Cylindrocarpon* Isolates for Cyclosporin Production.............................. 42

3.2 Confirmation of *T. niveum* Metabolites as Cyclosporins.............................. 45

3.2.1 Mass Spectrometric Analysis of *T. niveum* Metabolites..... 45

3.2.2 Biological Activity of *T. niveum* Metabolites.............................. 48

3.3 Inoculum Development.............................. 50

3.3.1 Determination of Optimum Conditions for Cyclosporin Production.............................. 50

3.3.2 Preculture Media and Inoculum Preparation.............................. 51

3.3.3 Optimization of the Inoculum for Production Culture......... 52

CHAPTER PAGE #

3.3.4 Effect of Spore Source on Cyclosporin Production........... 57

3.3.5 Spore Inoculum Effects on *T. niveum* Morphology.......... 59

3.4 Medium Development.. 66

3.4.1 Carbon Source Optimization.................................. 66

3.4.1.1 Determination of Optimum Carbon Source........... 66

3.4.1.2 Sorbose Preparation.. 70

3.4.1.3 Growth of *Tolypocladium niveum* on Tetradecane.. 72

3.4.2 Nitrogen Source Optimization................................ 74

3.4.2.1 Determination of Optimum Nitrogen Source.......... 74

3.4.2.2 Vitamin Assay Casamino Acids Preparation.......... 76

3.4.3 Optimization of Medium Salts for Cyclosporin Production.. 81

3.5 Procedural Considerations.. 85

3.6 Medium Supplementation.. 89

3.6.1 Directed Synthesis.. 89

3.6.2 Supplementation with Methyl Donors......................... 92

3.7 Time Course Experiments.. 92

3.7.1 Time Course Experiments at 27°C............................ 93

3.7.2 Time Course Experiments at 15°C............................ 98

3.8 Production of ^{14}C Labelled Cyclosporin................................ 101

3.9 Fermenter Production of Cyclosporin................................ 104

3.9.1 Stirred Tank Fermenter (10 L)................................. 107

3.9.2 Draught-Tube Airlift Fermenter (2 L)......................... 110

3.9.3 External Loop Airlift Fermenter (1 L)......................... 113

3.9.4 Comparison of Fermenter Designs............................. 117

CHAPTER PAGE #

4 DISCUSSION.. 121

5 REFERENCES... 129

LIST OF TABLES

TABLE

DESCRIPTION PAGE #

1 Amino Acid Composition of Seven Classes of Cyclosporin 6

2 Directed Synthesis of Cyclosporing Production 13

3 UAMH Strains of *Tolypocladium niveum* (Rostrup) Bissett (syn. *T. inflatum*) 29

4 Cyclosporing Production by *Tolypocladium* and *Cylindrocarpon* strains 43

5 Effect of Inoculum Biomass on *T. niveum* 58

6 Effect of Inoculum Size on Preculture Morphology and Cyclosporin Production 60

7 Cyclosporin Production on Different Media 67

8 Cyclosporin Production on Different Carbon Sources 69

9 Cyclosporin Production for Three Methods of Sorbose Preparation 71

10 Cyclosporin Production on Two Strains of *T. niveum* from Two Inoculum Sizes 73

11 Cyclosporin Production on a Variety of Nitrogen Sources 75

12 Cyclosporin Production on Different Lots of Vitamin Assay Casamino Acids 80

13 Effect of Phosphate Concentration on Cyclosporin Production 84

14 Effect of Initial pH and Phosphate Concentration on Cyclosporin Production 86

15 Directed Synthesis of Cyclosporing Production in Five *T. Niveum* Strains 90

16 Cyclosporin Production in Fermenters and Shake Flasks 118

LIST OF FIGURES

FIGURE	DESCRIPTION	PAGE #
1	Chemical Structure of Cyclosporin A	5
2	Sructure of 4'-Phosphopantetheine and a Model Showing the Operation of the Enzyme-Bound Cofactor	24
3	Calibration Curve of Cyclosporin Absorbance	34
4	Typical Chromatogram from HPLC Analysis of *T. niveum* Metabolites	36
5	Mass Spectrometric Analysis of *T. niveum* Metabolites	47
6	Comparison of Preculture Media and Inoculum Washing for Cyclosporin Production	54
7	Effect of Preculture Age on Cyclosporin Production	56
8	Effect of Preculture Inoculum Size on Cyclosporin Production	63
9	Aggregation of Germinating *T. niveum* Spores	65
10	Effect of Increasing Vitamin Assay Casamino Acids Concentration on Cyclosporin Production	78
11	Production of Cyclosporin and Nutrient Uptake over Time in Shake Flask Culture by *T. niveum*	97
12	Comparison of Mycelial Dry Weight, Cyclosporin Production, and Specific Cyclosporin Production over Time in Shake Flask Culture of *T. niveum*	100
13	Effect of Lowered Temperature on Cyclosporin Production in Three Strains of *T. niveum* in Shake Flask Culture	103
14	Uptake of ^{14}C methyl-methionine by *T. niveum* UAMH 2472	106

FIGURE DESCRIPTION PAGE #

15 Cyclosporin Production and pH of *T. niveum* UAMH 4594 over Time in a 10L Stirred Tank Fermenter 109

16 Comparison of *T. niveum* Growth and Cyclosporin Production in Shake Flask Culture and in a 1L Airlift Fermenter 116

Chapter 1: Literature Review and Objectives

1.1 Literature Review

The literature review for a project such as this must touch on a wide number of subjects. I have chosen to review the aspects of cyclosporin fermentations which are most closely related to the project, "Cyclosporin Production by *Tolypocladium niveum* strains", and which reflect my interests. To deal with these diverse subjects in an organized manner, the literature review will first describe the development of cyclosporin production from its initial isolation to the purification of the synthase enzyme and chemical synthesis of cyclosporin. Much of the work done in the field of cyclosporin production has been carried out by workers from Sandoz Chemical Company (Switzerland): the names Dreyfuss, Kobel, Traber, Borel, and Wenger appear frequently in any literature review or reference list of cyclosporin production or applications. Next, a brief section deals with the mode of action and the many clinical applications of cyclosporins as well as one other potential application in pest control. Secondary metabolites, and specifically peptide antibiotics, will then be discussed with particular emphasis on the related synthesis of the cyclic peptide antibiotics gramicidin S and enniatin. Finally, fermenter design and immobilization of fungal hyphae for metabolite production will be discussed along with the predominant method of cyclosporin detection, High Performance Liquid Chromatographic (HPLC) analysis.

1.1.1 Cyclosporin: A Fungal Metabolite

The literature relating to cyclosporin production is just 12 years old and began with the description of "new metabolites from *Trichoderma polysporum* " (Dreyfuss *et al.* 1976). The discovery of these compounds resulted from routine screening for new metabolites from microorganisms, specifically the fungus *T. polysporum* (Link ex Pers.)

Rifai by Sandoz. This organism was described as having a main conidiophore axis which measured 2.0-3.1 µm in width and usually terminated in a sterile flexous thread. The phialide-bearing branches, which could be either unbranched or branched, bore verticils of phialides and usually terminated in a single phialide. The phialides were swollen at the base and elongated into a long, thin neck, where the hyaline, subglobose to ellipsoidal, 2.0-3.1 x 1.5-2.0 µm conidia accumulated in small heads. The description of the strain's characteristics differed slightly from that of Rifai (1969) for *T. polysporum* in the width of the main conidiophore axis, the thin and long-necked phialides, and the relatively small conidia. A 1971 study by W. Gams (Gams, 1971) proposed a new genus for soil-inhabiting hyphomycetes with swollen conidiophores and amerosporous conidia borne in slimy heads. There were three species in the new genus *Tolypocladium* , *T. geodes*, *T. inflatum*, and *T. cylindrosporum* . A further 10 species were added to the genus in a study by J. Bissett (Bissett, 1983). In a comparison of mosquito-larvae parasitizing fungi, Sigler *et al.* (1987) discussed the nomenclature of *Tolypocladium*. The cyclosporin producing strain of *Tolypocladium* was originally named *Pachybasium niveum* (Rostrup). The cyclosporin-producing*T. polysporum* of Dreyfuss et al. (1976) was placed in the genus *Tolypocladium* as *T. inflatum*. Bissett (1983) determined that *P. niveum* (Rostrup) and *T. inflatum* (Gams) were the same taxon. The name given by Rostrup had priority in the new genus *Tolypocladium*, therefore the fungus was named *T. niveum* (Bissett, 1983). von Arx has attempted to rename the fungus *Beauveria nivea* (von Arx, 1986) though this designation does not appear to be well supported. The 1986 University of Alberta Microfungus Herbarium (UAMH) catalogue listed the *T. inflatum* species as *T. niveum*, the name used in all our work.

1.1.2 Chemical Structure of Cyclosporin

Cyclosporins are cyclic undecapeptides produced by *Tolypocladium* species which are widely used as immunosuppressants. Some chemical characteristics of cyclosporins were presented by Dreyfuss *et al.* (1976) including melting points (148-151°C for cyclosporin A and 152-155°C for cyclosporin C), optical rotations in both chloroform and methanol, chemical formulae, and molecular weights. The structure had been elucidated by chemical, spectroscopic, and crystallographic methods and was found to be that of a neutral cyclic peptide containing 11 amino acids, which are predominantly aliphatic, seven of which are N-methylated, contributing to the highly nonpolar nature of the compound (Figure 1). The molecule also contained a unique amino acid residue (2S,3R,4R,6E)-2-methylamino-3-hydroxy-4-methyl-oct-6-enoic acid. This amino acid, abbreviated L-C_9 had not been previously described and is designated as position 1 in numbering schemes. The only major difference in the structure of many of the classes of cyclosporins occurs at position 2 of the molecule: the original classes of cyclosporins (A-H in order of discovery) have differing amino acid residues at this position. For example cyclosporin A has L-α-aminobutyric acid at position 2 while cyclosporin C has L-threonine (Kobel and Traber, 1982);(Table 1). The more recent designation of twenty-five types of cyclosporin, some of which are chemically derived, differ in amino acid substitutions at positions 1, 2, 4, 5, 7, and 11 or may contain unmethylated peptide bonds in positions 6, 9, 10, or 11 (von Wartburg and Traber, 1986). The molecule also has hydrogen bonding at three points. The secondary structure is composed of an anti-parallel ß-pleated sheet conformation which contains three transannular H-bonds and is twisted. The remainder of the residues, 7-11, form an open loop (Wenger, 1984).

Figure 1 Chemical Structure of Cyclosporin A

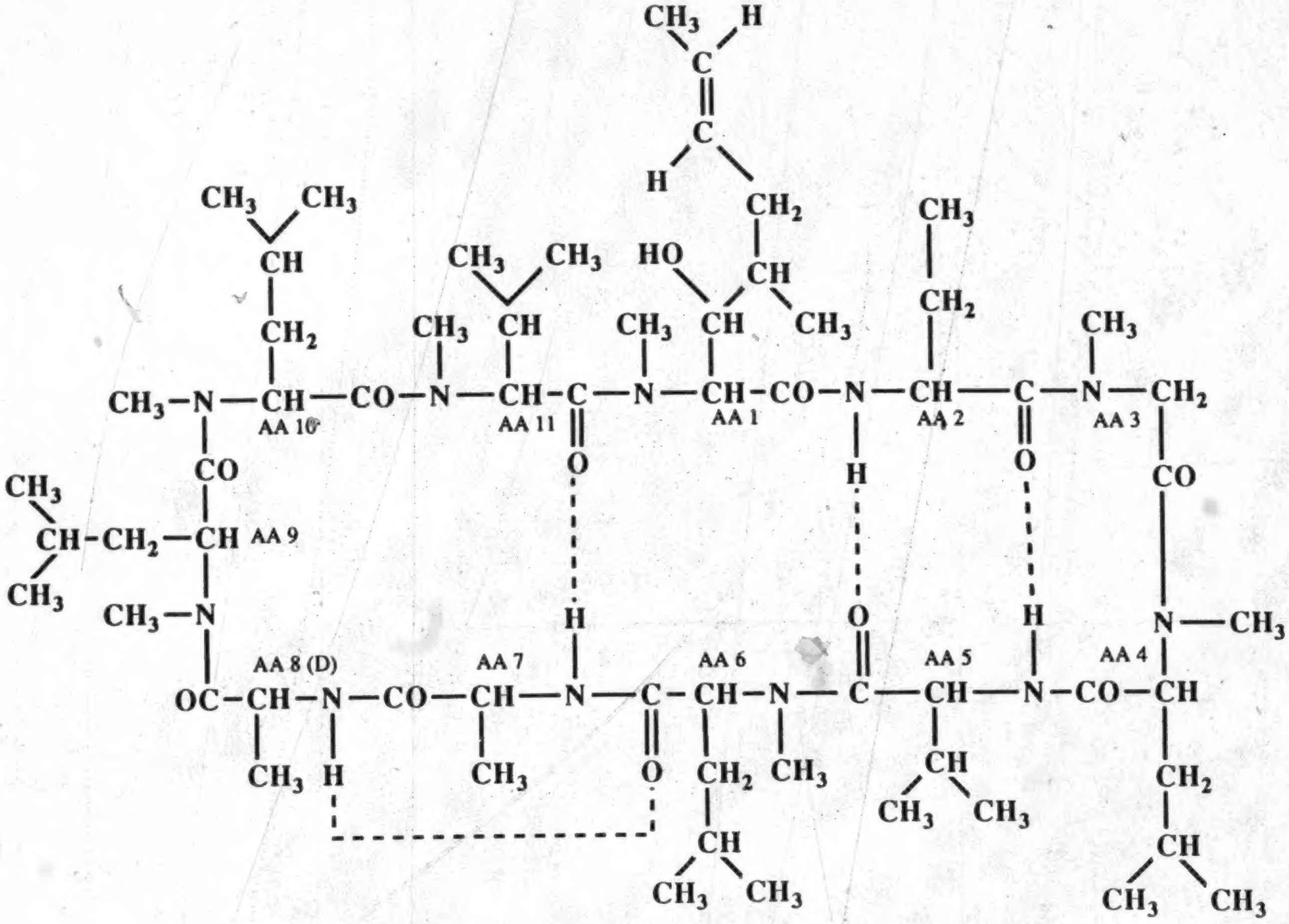

AA 1
AA 2
AA 3
AA 4
AA 5
AA 6
AA 7
AA 8 (D)
AA 9
AA 10
AA 11

Table 1 Amino Acid Composition of Seven Classes of Cyclosporin

Cyclosporin	Amino Acids										
	1[a]	2[b]	3	4[d]	5	6	7	8[f]	9	10	11[c]
A	L-C_9	L-Abu	Sar	L-MeLeu	L-Val	L-MeLeu	L-Ala	Ala	L-MeLeu	L-MeLeu	L-MeVal
B	L-C_9	L-Ala	Sar	L-MeLeu	L-Val	L-MeLeu	L-Ala	Ala	L-MeLeu	L-MeLeu	L-MeVal
C	L-C_9	L-Thr	Sar	L-MeLeu	L-Val	L-MeLeu	L-Ala	Ala	L-MeLeu	L-MeLeu	L-MeVal
D	L-C_9	L-Val	Sar	L-MeLeu	L-Val	L-MeLeu	L-Ala	Ala	L-MeLeu	L-MeLeu	L-MeVal
E	L-C_9	L-Abu	Sar	L-MeLeu	L-Val	L-MeLeu	L-Ala	Ala	L-MeLeu	L-MeLeu	L-Val
F	L-C_9 3-desoxy	L-Abu	Sar	L-MeLeu	L-Val	L-MeLeu	L-Ala	Ala	L-MeLeu	L-MeLeu	L-MeVal
G	L-C_9	L-Nva[c]	Sar	L-MeLeu	L-Val	L-MeLeu	L-Ala	Ala	L-MeLeu	L-MeLeu	L-MeVal
H	L-C_9	L-Abu	Sar	L-MeLeu	L-Val	L-MeLeu	L-Ala	Ala	L-MeLeu	L-MeLeu	D-MeVal

[a] L-C_9 is (2S,3R,4R,6E)-2-Methylamino-3-hydroxy-4-methyl-oct-6-enoic acid

[b] L-Abu is L-α-aminobutyric acid

[c] L-Nva is L-norvaline

[d] L-MeLeu is N-methyl-L-leucine

[e] L-MeVal is N-methyl-L-valine

[f] Position 8 Ala are all D

from Kobel and Traber, 1982

1.1.2.1 Detection of Cyclosporin

One consideration of primary importance to this study is the method of detection of cyclosporins in the *T. inflatum* cultures: the ideal method would be rapid, reproducible, and inexpensive. To this end the most appealing assay for cyclosporin would involve a rapid bioassay for semi-quantitative analyses. However cyclosporins have no effect on most bacteria and their fungicidal effect requires considerable quantities of the compound (Dreyfuss *et al.* 1976). Though several fungi are inhibited by cyclosporins, these include *Neurospora crassa* and *Aspergillus niger* (Dreyfuss *et al.* 1976), no bioassay utilizing these organisms has been described. Dreyfuss *et al.* (1976) also used thin layer chromatography (TLC) for the detection of cyclosporins. This method had the advantage of allowing recovery of the cyclosporin sample and for measurement of sample radioactivity in radioactive labelling experiments. The major limitation to this method was that it was neither rapid nor efficient. Another method of detection involved the extraction of the cultures, crystallization of the cyclosporins and checking physical properties such as melting point and optical rotation. This might explain why the minor classes of cyclosporins were not observed until HPLC detection became commonly used. None of these analyses were readily automated or cost-efficient. For these reasons other methods of cyclosporin quantitation were needed.

The first of these was a radioimmunoassay (RIA) for cyclosporins. A RIA was marketed by Sandoz for cyclosporin A and has been used almost exclusively for clinical applications. An exception to this was the use of RIA for cyclosporin analyses by Foster *et al.* (1983) as a confirmation of HPLC analysis. The analysis of plasma and urine samples for cyclosporins has been carried out routinely by RIA. However when compared to HPLC analysis it has been found that RIA tends to overestimate the quantity of cyclosporin present, presumably because it also detects metabolites of cyclosporins which have similar antigenic characteristics. A major drawback is the specificity of the assay in that RIA cannot distinguish between the classes of cyclosporins or their metabolites (Carruthers *et al.* 1983).

HPLC analysis eliminated both the problems of RIA, as the basis of separation and identification is the chemical properties of the molecule and its ultraviolet light absorbance (Carruthers *et al.* 1983). The HPLC analysis of cultures, plasma or urine samples involves an extraction with a non-polar solvent, subsequent drying of the extract, solution in another solvent, and injection into the HPLC system. The mobile phase of all cyclosporin HPLC analyses are solutions of acetonitrile, methanol, water, and occasionally phosphoric acid. There are a multitude of methods utilizing different types of reverse-phase columns, mobile phases, flow rates, and operating temperatures (Kreuzig, 1984). The cyclosporins themselves are resolved on the basis of polarity which is related to the substituent amino acids, their substitutions and methylations. The extraction procedure removes the majority of the culture components leaving a residue which is free of most interfering metabolites. The cyclosporins are easily resolved and can be positively identified by comparison with the elution times of known standards. The cyclosporins can easily be collected after analysis by a fraction collector. Detection of cyclosporins after separation is on the basis of ultraviolet absorbance at 214 nm due to the vibration of the peptide bond.

1.1.3 Biological Activity

Interest in cyclosporin has developed since the description of its unique and impressive immunosuppressive activity first observed by Borel *et al.* (1976). This initial interest has sparked research which has led to a number of diverse clinical applications. Additionally one unique entomological application has been observed for cyclosporin and a related compound produced by *Tolypocladium* species.

The biological effects of cyclosporin may be divided into four categories: antimicrobial, antiparasitic, antiinflammatory, and immunosuppressive. The antimicrobial activity of cyclosporins was found to be rather narrow. Only a few species of yeasts were sensitive and no inhibition of bacterial growth was observed. Some strains of Mucorales, ascomycetes, and deuteromycetes showed differing sensitivity against cyclosporins, this inhibition often manifests itself as deformation and branching of the growing hyphal tips or simply as a reduction in the growth rates of sensitive yeasts on solid medium. The aerial hyphae were not affected, nor were the spores or germination of the spores (Dreyfuss *et al.* 1976). There is no present clinical application for cyclosporin as an anti-fungal agent. However it was found that cyclosporin A inhibited the growth of the opportunistic pathogen *Cryptococcus neoformans* in a murine model. This basidiomycete is often observed in patients with decreased T-lymphocyte-mediated immune function including patients with acquired immune deficiency syndrome (AIDS). Mice treated *in vivo* with cyclosporin were able to survive indefinitely and were better able to clear the pathogen from their lungs. Cyclosporin administered *in vitro*, at levels equivalent to those found in the blood of the immunocompromised mice, was able to inhibit the growth of *C. neoformans*. The authors suggested that patients who receive cyclosporin A immunosuppressive therapy might be fortuitously protected against infections of *C. neoformans* (Mody *et al.* 1988).

The anti-parasitic activity of cyclosporin potentially has greater clinical usefulness. Cyclosporin has been shown to have a potent effect on schistosomiasis in a murine model, possibly through a host-mediated mechanism causing inhibition of the haemoglobinase and lowered protein levels in the schistosomatic worms. The result was a marked reduction in the male worm population and to a greater extent the female population (Bueding *et al.* 1981). Cyclosporin also has a dramatic effect on *Plasmodium falciparum* and other *Plasmodium* species, the parasites involved in malaria. In a murine model Thommen (1981) has demonstrated that optimal effects were obtained using subimmunosuppressive doses and that only a few doses were necessary to cure the mice. The best results were obtained when cyclosporin was administered during the parasitaemia and not at the time of infection. This indicated the drug acts directly on the plasmodium *in vitro* in contrast to the action on schistosomatic worms (Borel, 1982; Thommen, 1981).

Cyclosporin has been found to be effective, in preliminary reports, in many autoimmune diseases as an immunomodulator. Three significant examples of this immunomodulating effect are in posterior uevitis, Type I diabetes mellitus, and rheumatoid arthritis. In early animal trials, and later clinical human trials, occular inflammatory activity decreased and visual acuity increased once cyclosporin therapy started (Nusenblatt *et al.* 1982; Nussenblatt *et al.* 1983). In a study of Type I diabetes mellitus, cyclosporin administration within six weeks of diagnosis demonstrated a beneficial effect: over half the trial group were able to discontinue insulin use. After one year, the patients continued to do well though if cyclosporin therapy was discontinued insulin dependence developed (Stiller *et al.* 1984). The effect of cyclosporin on rheumatoid arthritis continues to be studied. Preliminary animal trials as well as double-blind placebo controlled studies in humans indicate an improvement in number of swollen joints, Ritchie articular index, and pain scores (van Rijthoven *et al.* 1985).

However the immunosuppressive activity of cyclosporin is by far the most important activity of the drug and this immunosuppressive effect appears to be selective for the T helper cells. Cyclosporin has been shown to be effective in a great number of transplant operations including those of the heart, liver, and kidney where tolerance has been induced across major histocompatibility barriers. In addition to cadaver kidney transplants where cyclosporin increases overall graft survival, cyclosporin has been used successfully in higher risk operations such as liver and heart-lung transplants. Prior to cyclosporin's use these operations suffered from a high incidence of complications and long term survival was poor (Belendiuk and Winter, 1986). Cyclosporin has also been found to be effective in modifying graft versus host disease in bone marrow transplantation without significant myelosuppressive effects (Poweles *et al.* 1978).

The major problem with cyclosporin therapy is the associated nephrotoxicity. The pattern of nephrotoxicity is idiosyncratic. There is a positive correlation between high level of abnormalities and higher doses of cyclosporin but these abnormalities can also be observed in a significant number of patients with lower cyclosporin levels. The precise therapeutic window for cyclosporin in the majority of autoimmune diseases is not known so precise alteration of the dosage is not easily carried out, in part because changes in renal function are not manifest until 6-8 weeks after the dose change. The final problem in this area is that the degree of immunosuppression based on standard laboratory parameters and clinical disease progression are poorly correlated (Belendiuk and Winter, 1986).

A novel application for cyclosporin and a related compound Tolypin was observed in the effect of these compounds on mosquito larvae. A fungus, which was later identified as *T.cylindrosporum*, was isolated from *Aedes australis* and *Aedes sierensis* by two different workers (Weiser and Matha, 1988a). The fungi killed the larvae of these two mosquito species by growth inside the hemocoel of the insect. This larvicidal effect was also observed for *T. geodes* and *T. inflatum* (=*T. niveum*) although fungal growth alone was not responsible for this killing effect. Another factor was isolated and found to be a water soluble, thermostable metabolite related to the fungal pekatrines named Tolypin (Wieser and Matha, 1988a). The identification of *Trichoderma polysporum* as *T. inflatum* (=*T. niveum*) resulted in a test of cyclosporin's mosquitocidal activity. A crude cyclosporin extract from *T. inflatum* (=*T. niveum*)did in fact demonstrate mosquitocidal activity but this effect was quite different from that of Tolypin. HPLC analysis indicated that Tolypin differed from cyclosporins and was eluted separately. Weiser and Matha (1988b) suggested that a synergistic effect between Tolypin and cyclosporin might occur under certain conditions. This mosquitocidal effect might be utilized as a biological test for cyclosporins.

1.1.4 Directed Synthesis of Cyclosporins

By 1982 the basic knowledge about the structure and immunosuppressive activities had been published. Scientists at Sandoz had described a protocol for the production of cyclosporins. The directed biosynthesis of actinomycins had been reviewed by Katz and Demain (1977) and was now attempted by Kobel and Traber (1982) for cyclosporins. The fungus *T.inflatum* (=*T. niveum*) used in these experiments was a mutant derived from the parent *T. inflatum* NRRL 8044 strain. As cyclosporins of the classes A, B, C, D, and G differ in chemical structure only at the second amino acid residue, adding an excess of a particular substituent amino acids to the culture might direct synthesis to the desired class of cyclosporin. Kobel and Traber were indeed able to direct synthesis. This was not complete in all cases but the less commonly produced classes of cyclosporins, such as D and G, were now produced in good yield (see Table 2). This ability to direct synthesis reflects the low specificity in the non-ribosomal biosynthesis of peptides in eukaryotes.

1.1.5 Radioisotope Labelling

To better understand the assembly and synthesis of cyclosporins a series of radioactive labelling experiments were carried out by Kobel, Loosli, and Voges (1983). Their experiments initially used ^{14}C-labelled substrates to determine the best precursors for later ^{13}C-labelling for NMR studies. ^{14}C-Methyl labelled methionine proved to be the best precursor for the production of radiolabelled cyclosporins. Experiments were carried out using both ^{14}C- and ^{13}C-labelled acetate and ^{14}C- and ^{13}C-methyl methionine to determine the precursors necessary for complete cyclosporin synthesis. The results indicated that the methyl groups involved in the N-methylation were derived from the methyl group of methionine. The unique amino acid residue at position 1, abbreviated L-C_9 , was found from ^{13}C-NMR studies to be derived from the head to tail condensation of four acetate units. Further studies involving radioactive labelled precursors were carried out by Zocher

Table 2 Directed Synthesis of Cyclosporin Production

Precursor 8 g/L	Mycelial Dry Weight (g/L)	Total Cyclosporins (mg/L)	Single Compounds (% total cyclosporins) A	B	C	D	G
0=control	19	131	77	--	23	--	--
DL-α-Abu	19	249	100	--	--	--	--
L-Ala	23	113	51	13	36	--	--
L-Thr	22	672	59	--	41	--	--
L-Val	19	743	43	--	20	37	--
L-Nva	23	260	9	--	--	--	91

α-Abu= α-aminobutyrate

Nva= norvaline

from Kobel and Traber, 1982

et al. (1983). These experiments involved feeding ^{14}C- radiolabelled precursor amino acids, L-valine, L-leucine, D and L-alanine, glycine, and methyl -labelled methionine. The results indicated that a particular amino acid was incorporated only at its appropriate position in the cyclosporin molecule. The model of cyclosporin assembly, in which the methylations occur simultaneously after assembly of the molecule from donor methionine, was supported not only by the incorporation of activity when ^{14}C-methyl labelled methionine was added, but also by the lack of incorporation of ^{14}C-methyl labelled sarcosine (N-methyl glycine). This agreed with the results of Kobel *et al.* (1983). It was suggested that the synthesis of cyclosporin might be similar to that of enniatin, where a multifunctional enzyme carries out N-methylation of the constituent amino acids after their activation as enzyme-bound thioesters (Zocher and Kleinkauf, 1978).

1.1.6 Fermenter Design and Fermenter Production of Cyclosporin

Production of industrially important products must be carried out on a larger scale than shake flasks. For this reason fermenters are used to increase the size of the fermentation and to control the many associated variables. The growth of fungal cultures in fermenters requires different considerations from bacterial cultures.

The physical characteristics of fungal stirred tank and stationary cultures are very different from those of bacteria or yeast (Burkholder and Sinnott, 1945). This is due to the morphology of filamentous fungi and the resulting non-Newtonian properties of these fermentations including uneven mixing of the culture. The filamentous fungi place a higher demand on the fermenter system (mass transfer, heating, and mixing problems) as the cultures have a higher viscosity due to the filamentous nature of the fungi. These demands can be overcome by changing the engineering variables of the fermentation such as increasing impeller speed (van Suijdam and Metz, 1981). However a change in a physical parameter often results in a change in the fermentation, increased impeller speed would result in shorter hyphal growth which would reduce viscosity of the culture. The amount of impeller energy input would be related to the tensile strength of the hyphae and would obey the law of diminishing returns.

An alternative to the filamentous morphology of many of the industrially important fungi is either a pelletized morphology or the immobilization of the fungi in a matrix. The most important advantage of these forms is that the viscosity of the culture is reduced though problems in reduced oxygen transfer and sampling may occur.

Mycelial pellets have been used in both stirred tank and airlift fermenters to produce a wide variety of products (Martin and Waters, 1952; Steel *et al.* 1954; Clark, 1962; Konig *et al.* 1982; Kloosterman and Lilly, 1985; Martin and Bailey, 1985; Wase *et al.* 1985). The factors affecting pellet formation have been well documented including agitation, growth medium, pH, oxygen tension, polymer additives, and growth rate (Ward and Colotelo, 1960; Whitaker and Long, 1973; van Suijdam *et al.* 1980; Schurgel *et al.* 1983). The size of inoculum has been recognized as being one of the most important factors in pellet formation and it can be stated that large inocula generally result in a filamentous morphology while small inocula produce pelletized morphology (Metz and Kossen, 1977).

Mycelial pellets have been investigated for oxygen mass transfer (Ho *et al.* 1984) and it has been found that oxygen depletion in the pellet or bead centre will cause autolysis of the fungus resulting in a hollow centre (Metz and Kossen, 1977). Oxygen depletion in the pellet is not limiting until a critical diameter is reached whereby a gradient of oxygen diffusion is established with the centre finally receiving none. Immobilized pellets have been examined microscopically to determine the effect of κ-carrageenan concentration on mycelial growth inside the beads (Deo *et al.* 1983). It was found that carrageenan concentrations over 4% resulted in peripheral growth.

Use of pelletized or immobilized fungal cells has been reported for a great number of applications. These include the production of citric acid (Tsay and To, 1987), enzymes

(Kobuku *et al.* 1978; Kobuku *et al.* 1981; Frein *et al.* 1982; Wase *et al.* 1985; Suter *et al.* 1986; Kirk *et al.* 1986), ethanol (Linko *et al.* 1981; AiresBarros *et al.* 1987; Godia *et al.* 1987), gibberellic acid (Kahlon and Malhotra, 1986), and cyclosporin (Foster *et al.* 1983). Recent uses have included use of pelletized or immobilized genetically-modified bacterial or fungal cells to produce extracellular foreign proteins (Das and Schultz, 1987; Younes *et al.* 1987).

Cyclosporin production in a (small scale) external loop airlift fermenter with shake flask cultures using carrageenan-entrapped and free mycelia of *T. inflatum* NRRL 8044 had been shown by Pasutto's group (Foster *et al.* 1983). An airlift fermenter was used because of its simple configuration, cost effectiveness, and hydrodynamic character. Immobilization of the mycelium in 3% carrageenan avoided the problems associated with maintaining the pellet morphology during the fermentation. Cyclosporin production levels ranged between 240 mg/L cyclosporins in the fermenter to 300 mg/L cyclosporins in the free mycelial shake flask cultures. These production values were detected using both HPLC-UV absorbance analysis and the commercially available RIA with a positive correlation between the values detected. Foster *et al.* (1983) also attempted to operate the airlift fermenter using an inoculum of free mycelia but found that under their conditions the entire inoculum was carried onto the walls of the fermenter by the bubbles and effectively removed from the fermentation. The large amount of foam present in both the shake flasks and fermenter was thought to be an qualitative indication of cyclosporin production since the medium was designed to be low foaming. Therefore it was likely that foam production was a result of fungal action. The authors concluded that the fermenter conditions had not yet been optimized.

1.1.7 Medium Composition and Growth Conditions

Since the first production studies, a large number of different media and growth conditions have been tested for cyclosporin fermentations. The organism used throughout most of the literature has been the original strain of *T. inflatum* (=*T. niveum*) which is designated either as NRRL 8044 or DSM 915 (deposited as *Trichoderma polysporum*). These strains, or mutants thereof, were used exclusively (Dreyfuss *et al.* 1976; Kobel and Traber, 1982; Billich and Zocher, 1987) including the *T. inflatum* ATCC 34921 strain used by Agathos *et al.* (1986 and 1987). In all of these studies, cyclosporin production involved preculturing a spore suspension in a rich medium to generate biomass then transfer to a more nutritionally-limited medium in which the secondary metabolite was produced. These limited media have used a great range of carbon and nitrogen sources including glucose (Foster *et al.* 1983), sucrose and malic acid (Kobel and Traber, 1982), maltose (Kobel *et al.* 1983), corn steep molasses (Zocher *et al.* 1984), caseinpeptone (Dreyfuss *et al.* 1976), vitamin assay casamino acids (Foster *et al.* 1983), and sodium nitrate (Foster *et al.* 1983). These media have also used a great variety of salts, trace element solutions, and vitamin supplements (Kobel and Traber, 1982). The growth conditions and times of fermentations also varied greatly from shake flask cultures to industrial scale fermenters (Dreyfuss *et al.* 1976).

Final production of cyclosporin has also varied. Dreyfuss *et al.*(1976) described production of 200-300 mg/L cyclosporins in a glucose-caseinpeptone medium. Kobel and Traber (1982) noted production of 113-750 mg/L cyclosporins in a sucrose-malic acid medium supplemented with substrate amino acids. Foster *et al.* (1983) were able to produce 300 mg/L cyclosporins in a dextrose-vitamin assay casamino acids medium. Agathos *et al.* were able to show only 150 mg/L cyclosporins using sorbose-bactopeptone medium (with *T. inflatum* ATCC 34921). This range of both medium components and total production presented problems in establishing the methodology and media with a new strain of *Tolypocladium* to yield high, reproducible levels of cyclosporin.

1.1.8 Nutritional Studies

The first published comprehensive study of the nutritional requirements for cyclosporin synthesis was by Agathos *et al.* (1986). They described attempts to optimize cyclosporin production by varying the carbon source and nitrogen source in the medium. Carbon and nitrogen components were varied in a base medium of potassium phosphate and potassium chloride. Fungal growth and cyclosporin production were determined after 10 days at 27° C in shake flask culture. An investigation of 22 carbon sources, including pentoses, hexoses, disaccharides, polysaccharides, and organic acids showed certain carbon sources supported higher cyclosporin levels and these were further tested to determine their optimum concentration for cyclosporin production. The results indicated that the carbon sources which were highly favorable for biomass production did not necessarily yield optimum secondary metabolite production: this has been observed in a wide variety of antibiotic fermentations including ß-lactams and amino-glycosides (Demain *et al.* 1983). This phenomenon was observed for increasing concentrations of glucose and maltose but not necessarily for other carbon sources tested. The conclusion reached was that each carbon source ought to be tested separately to determine its optimum concentration for cyclosporin production. Agathos'(1986) group also determined that the optimum carbon source was 2% sorbose.

The batch fermentation kinetics were also examined. Agathos *et al* . determined that the production of cyclosporins was essentially complete after ten days, beyond this time there was considerable hyphal fragmentation and autolysis. The production followed the growth curves described by Dreyfuss *et al.* (1976) where cyclosporin production was slow initially as the carbon source was rapidly taken up and the production increased during the slower growth phase. Another aspect of batch kinetics which was investigated involved fed-batch culture. Cultures started on sorbose as the carbon source were then supplied with maltose on day 8. Following a 3-4 day lag cyclosporin production resumed resulting in final production which was markedly higher than for either carbon source alone.

The nitrogen source was also investigated by Agathos (1986) as a possible means to increase cyclosporin production. The inorganic nitrogen sources tested failed to yield satisfactory cyclosporin levels. The complex nitrogen sources proved to be superior, bacto-peptone yielding the highest cyclosporin levels. A preliminary study into the effect of temperature on cyclosporin production indicated that cyclosporin production was higher at 15°C than at 27°C, the temperature which had previously been used by all other investigators. Preliminary studies for further genetic studies were also carried out including successful protoplast formation and determination of antibiotic sensitivity where it was found that *T. inflatum* (=*T. niveum*) was virtually insensitive to chloramphenicol, mycostatin, or cycloheximide. This insensitivity was suggested to be indicative of high resistance of permeability to the antibiotics tested.

1.1.9 Chemical Synthesis of Cyclosporin

The total synthesis of cyclosporins A and H as well as several analogues has been carried out by Wenger at Sandoz (Wenger, 1984 and 1985). The rationale for synthesis involved the assembly of a linear cyclosporin molecule and cyclization by forming a peptide bond between position 7 L-alanine and position 8 D-alanine. This strategy was used to take advantage of the fact these residues are non-methylated and bond formation is less difficult between amino acids which are not N-methylated. The synthesis of cyclosporins and analogues is now readily carried out and could serve as a powerful research tool in determining the biological activity and the structure-activity relationships. These relationships indicate that the amino acid L-C_9 is very important in biological activity as are positions 1, 2, 3, and 11 of the molecule (Wenger, 1985; Quesniaux, 1988).

1.1.10 Cell Free Synthesis of Cyclosporin

Another aspect of the production of cyclosporins that has also recently been investigated is the cell-free enzymatic synthesis. Work in this direction has been carried out by Zocher *et al.* (1986) describing the partial purification of a multifunctional enzyme from a nitroso-guanidine-derived mutant of *T. inflatum* DSM 915 (deposited as *Trichoderma polysporum*). An enzyme fraction with an apparent molecular weight of 700 000 daltons was purified from *T. inflatum* (=*T. niveum*) extracts. The results indicated that this enzyme was involved in the biosynthesis of the cycloundecapeptide. The evidence comes from the finding that the enzyme catalyzed the activation of amino acids present in the in the peptide chain as thioesters via the corresponding adenylates. The enzyme also catalyzed the N-methylation of thio-esterified amino acids present in cyclosporin A. The results indicated a similarity to the N-methylation previously shown in cyclodepsipeptide synthesis (Zocher *et al.* 1982; Peeters *et al.* 1983). The formation of the diketopiperazine *cyclo*-(D-ala-Meleu) strongly resembles that of *cyclo*-(D-phe-pro) in the synthesis of gramicidin S (Kurahashi, 1961). Significant amounts of the *cyclo*-(D-phe-pro) are produced only when D-phe and L-pro are present as the substrate amino acids of gramicidin S synthase (Otani, 1966). The inability of the enzyme to produce the entire cyclic undecapeptide was unexplained but the reason might be *cyclo*-(D-ala-Meleu) synthesis takes place as the non-enzymatic cyclization of the enzyme-bound thio-esterified dipeptide.

The work on purification of this enzyme continued and was described in the 1987 communication by Billich and Zocher (1987). Enzyme purification was carried out as described in the 1986 paper and the resulting extract was found to synthesize the complete cyclosporin A molecule. Why the enzyme purified in the 1986 paper (Zocher *et al.*) did not synthesize cyclosporin is unclear. The synthesis of particular cyclosporin classes could be directed by the addition of the appropriate substrate amino acid. From these experiments it was clear that the enzyme exhibits different affinities for the exchangeable amino acids (particularly at position 2). For example the incorporation of L-ala into position 2 resulting in cyclosporin B is poor, while cyclosporin G (formed by substituting L-nor) was obtained in quantities comparable to cyclosporin A (Billich and Zocher, 1987). These findings were in agreement with Kobel and Traber's findings on directed synthesis (1982). The authors suggested the production of specifically labelled cyclosporins with high specific activity for pharmacokinetic studies and the production of cyclosporins in an enzyme reactor as possible applications of this cell-free system.

1.1.11 Peptide Antibiotics as Secondary Metabolites

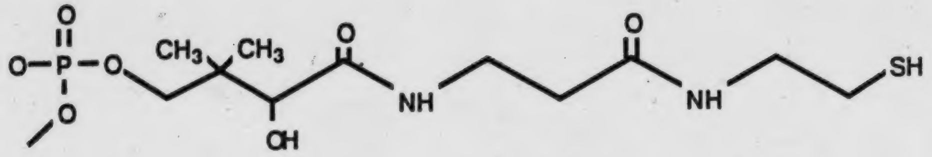
The term, secondary metabolites, has been defined in several ways. All definitions tend to have a number of properties in common: complex chemical composition, production by involved, often lengthy pathways, relatively free of turn-over, difficult to integrate into the intermediate, general physiology of the producer, produced only in the idiophase, not in the trophophase (nomenclature according to Bu'Lock *et al.* 1965), and are unnecessary for the growth of the producing organism (Campbell, 1984). Many of the peptide antibiotics are classified as secondary metabolites because the enzymes necessary for their production are repressed during growth and derepressed during the transition from trophophase to idiophase. These peptide antibiotics, which include alamethicin, bacitracin, enniatin, edeine, linear gramicidin, gramicidin S, polymyxin, tyrocidin, and cyclosporin, are all polypeptides which are produced by nonribosomal peptide bond formation. The enzymes which carry this out in each particular producing organism are generally considered to be multifunctional enzymes (Kleinkauf and von Dohren, 1983). A number of general functions are observed for all these multifunctional enzyme systems. The first of these common functions is the amino acid activation and enzyme aminoacylation (these two functions have been uncoupled for Gramicidin S synthases 1 and 2). The second general function involves the racemization of the amino acids. This process is poorly understood, however there is evidence of D-amino acids being activated when the L-form is required in the final product. This is indicative of a certain nonspecificity in the activation process. The third general function in the process of nonribosomal polypeptide formation is the actual peptide formation. The peptide bonds are formed in initiation, elongation, and termination reactions (Kleinkauf and von Dohren, 1983).

The general principle in elongation is "head growth" (Lipmann, 1968) where the activated carboxyl group of a donor amino acid or peptide reacts with the amino group of an enzyme-bound acceptor amino acid. The central role in elongation reactions is attributed to the cofactor 4'-phosphopantetheine which serves a transport function in many systems involving activated carboxyl groups. The 4'-phosphopantetheine cofactor is covalently attached to the enzyme complex. A model for elongation of the growing peptides has been proposed which shows the long cofactor swinging around the enzyme complex with the growing peptide chain bound by a terminal sulfhydryl of the phosphopantetheine. As the cofactor moves to the next enzyme-bound activated amino acid that amino acid is bound to the preceding amino acid in the chain resulting in chain elongation (Figure 2). This system of nonribosomal peptide bond formation has been given many names including a protein template mechanism (Lipmann, 1971), a thiotemplate mechanism (Laland and Zimmer, 1973), and a multienzyme thiotemplate mechanism (Kurahashi, 1981).

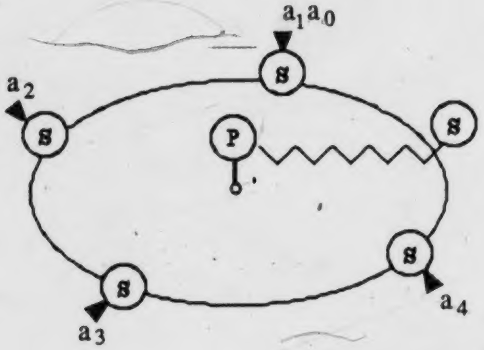
The general chemical properties of the peptide antibiotics are all quite similar. Many of them are cationic surfactants (Gramicidin S, tyrocidins, and polymyxins), cation binders (bacitracins) or neutral surfactants (linear gramicidins), and their biochemical properties are related to their chemical properties. Most of the these peptide antibiotics are membrane-active: several form channels or transport cations (linear gramicidin, Gramicidin S, and bacitracins), several are enzyme inhibitors (Gramicidin S and bacitracins), and some affect DNA-binding (tyrocidins). These chemical or biochemical properties are

Figure 2 Structure of 4'-Phosphopantetheine and a Model Showing the Operation of the Enzyme-Bound Cofactor

This figure demonstrates amodel for the elongation of the peptide chain in non-ribosomal peptide synthesis. The enzyme activated amino acids are bound to the enzyme complex at sulfhydryl groups. As the cofactor swings by these activated amino acids they bind to the sulfhydryl of the cofactor and become bound to the amino acid which was previously bound to the cofactor. Through this mechanism the peptide elongates to completion. eg. $S\text{-}a_4a_3a_2a_1a_0$



4' phosphopantetheine



shared by a large number but not all peptide antibiotics (Kleinkauf and von Dohren, 1983).

Cyclosporin appears to be one of these peptide antibiotics. From the work of Dreyfuss *et al.* (1976) cyclosporins appear to be produced as secondary metabolites, in that their production becomes readily apparent after trophophase has ended. The work of Zocher *et al*. (1986) and Billich and Zocher (1987) gave preliminary results indicating that the cyclosporin "synthase" is a multifunctional enzyme similar to that described for enniatin synthesis by Zocher *et al.* (1982, 1983) as well as the peptide antibiotics described above. At present ~~the~~ detailed biochemistry and enzymology of cyclosporin synthesis is at present not described as well as for other peptide antibiotics such as enniatin (Zocher *et al.* 1982; Zocher *et al.* 1983), beauvericin (Peeters *et al.* 1983) or Gramicidin S (Laland and Zimmer, 1973; Frøyshov *et al.* 1978).

1.2 Objectives

From the literature review it is evident that no single protocol has been established for the production of cyclosporin from *T. inflatum*. Initially this project was conceived as a collaboration with a group in the Department of Chemistry at the University of Alberta who were unable to reproduce literature values of cyclosporin production using the methodology of Kobel and Traber (1982) and *T. inflatum* NRRL 8044 (=*T. niveum*). The assistance of our laboratory was requested because of a familiarity with techniques of fungal culture and metabolite isolation.

Preliminary experiments by other workers in this laboratory confirmed the results of the group in the Chemistry Department that cyclosporin production was inconsistent. This thesis topic was assigned as a systematic analysis of all aspects of the methodology involved in cyclosporin production. This included the following objectives:

1.2.1 Screening a number of *T. niveum* isolates, using previously established methodology, to determine if *T. inflatum* NRRL 8044 (=*T. niveum*)was the best organism for cyclosporin production.

1.2.2 Analysis of the methodology for cyclosporin quantitation, including extraction and the methods for cyclosporin determination.

1.2.3 Survey of the medium components to select the most productive with the chosen strain.

1.2.4 If, and when, these parameters had been determined, to attempt to scale up from shake flasks to laboratory scale stirred tank or airlift fermenters.

1.2.1 Screening for Cyclosporin-Producing Strains of *T. niveum*

The organism which had been obtained from the Northern Regional Reasearch Laboratory *T. inflatum* NRRL 8044 (=*T. niveum*) previously produced only low levels of cyclosporin (10-20 mg/L) in this laboratory using published methods, medium composition and growth conditions of Foster *et al.* (1983). Therefore a number of *T. niveum* strains were collected to be screened to determine if higher cyclosporin levels were produced than the widely used NRRL 8044 strain. Ten strains of *T. niveum* and one strain of *Cylindrocarpon lucidum* were obtained from the University of Alberta Microfungus Herbarium (UAMH) and compared to the *T. inflatum* (=*T. niveum*) NRRL 8044 strain. The strains producing the greatest quantities of cyclosporin would be selected and used for further work.

1.2.2 Development of Methods

A number of methods used for the growth of *T. niveum* and the analysis of cyclosporin have been used. A methodology which produces reproducible levels of cyclosporin content must be found. Variables in growth, including the inoculum source and age, in analysis, such as extraction procedures and solvents, and methods of cyclosporin determination must be standardized to minimize any experimental variability.

1.2.3 Medium Development

Once strains which produce the high levels of cyclosporin have been found, the objective of this research is to optimize the production of cyclosporin to the highest levels possible. The simplest method to optimize production is by altering the growth conditions of the fermentation such as inoculum size and age, fermentation time, medium composition, spore source, temperature, in fact almost any variable the organism is subjected to can be altered in an organized manner to find the conditions best suited to cyclosporin production. This study was carried out in batch shake-flask culture and consumed the greatest amount of effort. Once the optimum conditions for production were established, yielding consistent and reproducible results, and a growth curve defining the parameters of the fermentation was found, then further work involving scale-up carried out and the possibility of mutagenesis entertained.

1.2.4 Scale Up of Cyclosporin Production

The work in scale-up of the fermentation involved moving from shake flask culture to one of the fermenter designs available: 10L stirred tank Microferm™, 1L external loop airlift , and 2L draught-tube airlift fermenters. These designs were used not only to determine if cyclosporin was produced, but which fungal morphology produced the highest cyclosporin levels. Once the most effective design had been determined, a growth curve similar to that determined for shake flask culture was established. The result of this research was a consistent, reproducible protocol for both shake flask and fermenter production of cyclosporin.

Chapter 2: Methods and Materials

2.1 ~~Microbi~~ological

Nine strains of *Tolypocladium niveum* and one strain of *Cylindrocarpon lucidum* were obtained from UAMH. The *T. niveum* strains included: UAMH 2472, UAMH 2880, UAMH 4002, UAMH 4553, UAMH 4594, UAMH 4740, UAMH 4828, UAMH4900, UAMH 4901 and *C. lucidum* UAMH 4898 (see Table 3). The *T. inflatum* NRRL 8044 was obtained from the Northern Regional Research Laboratory Peoria, Ill., U.S.A. courtesy of Dr. J.C. Vederas, Deparment of Chemistry, University of Alberta.

The freeze dried preparations were reconstituted with distilled water and grown on 2% malt extract-0.4% yeast extract agar plates at 27°C. All UAMH strains were received on agar plates then subcultured on cereal agar slants. The stock slants were kept at -20°C and routinely subcultured every two years. Working cultures were kept on malt-yeast extract agar and stored at 4°C until used. Agar plates were used for working cultures until glycerol spore stocks of the five highest cyclosporin producing strains were prepared by spreading malt-yeast agar plates with spores from the original plates received from UAMH. These plates were grown at 27°C to confluent sporulation. The plates were then scraped and the spores suspended in sterile 20% glycerol to give 10^8 spores/mL. The glycerol suspension was then placed in sterile Nunc™ tubes (1mL glycerol suspension per tube), frozen to -75°C and maintained at this temperature. The glycerol suspensions were quantitated by plating dilutions of the suspensions on malt extract-yeast extract plates and counting the resulting singler spore colonies.

2.2 Growth in Submerged Culture

Fungal spores were inoculated into a preculture composed of one of several media including 1% malt extract-0.4% yeast extract medium; Foster's medium: 1.6% glucose,

TABLE 3 UAMH Strains of *Tolypocladium niveum* (Rostrup) Bissett (syn.*T. inflatum*)

2472 water sample, Whitehorse, J.W. Carmichael, 15-05-65.

2880 soil under *Pinus contorta*, C horizon, Kananaskis, Alta.,P. Widden, 11-67/G.C. Bhatt alpha 77 (=DAOM 167132) cyclosporin A.

4002 muskeg soil, Tuktoyatuk, N.W.T. S. Davies (H-46-F), 1976 /S. Davies 46 utilization of aliphatic hydrocarbons. Davies and Westlake, Can. J. Microb. 25:146-156, 1979.

4553 soil, Hardanger Vidda, Norway, J. Hansen, 1974 /B. Foster (=NRRL 8044=ATCC 34921= CMI 187376?=UAMH 4740)

cyclosporin A & C in submerged culture. Dreyfuss et al., Eur. J. Appl. Microbiol. 3:125-133, 1976.

4594 (N) washed organic particle, alpine meadow, 2530 m, Mt. Allen, Kananaskis, Alta., J. Bissett, 07-05-69/M. Goettel (=DAOM 167322=ATCC 42437)

4740 soil, Hardanger Vidda, Norway, J. Hansen, 1974/J. Vederas (=ATCC 34921=NRRL 8044=CMI 187376?=UAMH 4553)

cyclosporins A, B, C, D, E. Helv. Chim. Acta **59**:1075-1092. 1976 & ibid. **60**:1568-1578, 1977

4828 (T *Tolypocladium inflatum*) humus alpine soil, Obergurge Oetztal, Austria, W. Gams, 1959/B. Foster (=DAOM 64352=CBS 824.70)

4900 mite surface *Mycobates* sp., Frobisher Bay, Baffin Island, V. Behan (C), 1976/B. Foster (=DAOM 160594)

4901 humified organic material under spruce-fir forest, Luscar Alta., S. Visser, 08-76/B. Foster (=DAOM 167175)

from University of Alberta Microfungus Herbarium catalogue, 1987

1.0% casamino acids, 0.5% $NaNO_3$, 0.5% NaCl, 0.25% KH_2PO_4, 0.05% $MgSO_4 \cdot 7\ H_2O$ (Foster *et al.* 1983) or Agathos' medium: 2.0[illegible]orbose, 1.0% vitamin assay casamino acids, 1.0% KH_2PO_4, and 0.5% KCl, (Agathos *et al.* 1986; =SVA medium). The precultures were incubated on a gyrotary shaker (200 r.p.m.) at 27°C for 72-180 h. The precultures were homogenized for 10 s at full speed in a Sorvall Omnimixer and inoculated into production medium. The inoculum size (1-10%) and age were dependent on the experiment. The composition of the production medium also varied as described in the results section and was usually derived from either Foster's medium or Agathos' medium (see above). Production cultures were incubated for 15 days, unless otherwise indicated, at 27°C on a gyrotary shaker (200 r.p.m.). The production cultures were homogenized for 10 s at full speed in a Sorvall Omnimixer, made up to original volume with distilled water (where indicated) and samples were analyzed for cyclosporin content, pH and biomass. In the case of cyclosporin production in fermenters the precultures were prepared as described but the inoculation was made into the fermenter vessel.

2.3 Extraction of Cyclosporins from Culture Homogenates

The cyclosporins were extracted from the cultures by the following method. Equal volumes of culture and ethyl acetate were added to a 65 mL medicine bottle. The suspension was placed on a reciprocal shaker and shaken overnight (minimum time is 12 h). The resulting suspension was centrifuged at low speed to break up the emulsion and the organic phase collected. The organic phase was dried with anhydrous sodium sulfate and a 1 mL aliquot taken for analysis. This aliquot was taken to dryness with forced air, taken up in HPLC grade acetonitrile, and analyzed for cyclosporin content by HPLC.

2.3.1 Comparison of Extraction Methods

The choice of extraction method was made after comparison with other methods and solvents. Ethyl acetate (Foster *et al.* 1983) and butyl acetate (Agathos *et al.* 1986) had been used to extract cyclosporins as had methanol (Kobel and Traber, 1982). Comparison of ethyl and butyl acetate extractions using equal volumes of solvent and culture showed no difference in extraction efficiency. Because the cost of ethyl acetate is just over one-half that of butyl acetate, ethyl acetate was chosen. Later experiments compared the cyclosporins extracted with equal volumes of ethyl acetate and culture (overnight) to 9:1 extractions with methanol:culture. Methanol extractions gave less consistent cyclosporin values and more complex HPLC profiles, therefore ethyl acetate extraction of cyclosporins was continued.

2.4 High Performance Liquid Chromatographic Analysis for Cyclosporins

The primary method of quantitating cyclosporins produced by the fungus was by HPLC analysis. The separation and quantitation of cyclosporins were carried out using a Waters system: a model 501 HPLC pump, U6K Universal injector, a TCM (temperature control module) and Oven, and a model 441 Absorbance Detector (equipped with zinc/cadmium lamp and appropriate 214 nm frits and filters), coupled with a Hewlett Packard 3392A Integrator. Separation was performed on a reverse phase Brownlee RP-8 analytical column with an RP-8 NewGuard column. The system was run isocratically with a flow rate of 2.0 ml/min., oven temperature 72°C, mobile phase acetonitrile: methanol: water (47:20:33). Injections of a constant 10 μL volume were made with a Hamilton (Waters 802 RN 25 μL) syringe using appropriate dilutions. As the number of injections increased, the resolving power of the column decreased resulting in shorter elution times. To remedy this, the columns were regenerated by periodically flushing with 300 mL HPLC grade water followed by 300 mL HPLC grade methanol. However when regeneration proved unsuccessful, the column was replaced (200-250 h or 500 injections).

A calibration curve was constructed (Figure 3) and the slope was determined to be 0.166 μg cyclosporin/10^6 area units. Cyclosporin A standards were run each day to ensure the peak area determined fell on the standard curve. Multiple injections were carried out until duplicates with the less than 10% difference between the total peak areas were obtained. Unless specified, cyclosporin production represents the total cyclosporin produced (Cyclosporin A+B+C). A typical chromatogram is shown in Figure 4.

2.5 Bioassays for Cyclosporin Activity

Cyclosporin had been shown to have an inhibitory effect on *A. niger*. (Dreyfuss *et al.* 1976). Two methods were devised to utilize this sensitivity in a bioassay. The first method involved removing plugs of agar and mycelium (diameter=5 mm, depth=7-8mm) from sporulating plates of *T. niveum*. These plugs were placed on a fresh malt-yeast extract plate and incubated 24 h at room temperature. Standards were prepared by adding standard amounts cyclosporin A in acetonitrile dropwise to the agar plugs and preincubating. After this preincubation the plates were seeded with spores of *A. niger*. Seeding was done by placing a plate of confluently sporulating *A. niger* directly over the test plate. The *A. niger* plate was then lightly tapped to knock the *A. niger* conidia free which grew as a lawn except in the regions of inhibition. The diameters of the zone of growth inhibition and zones of inhibited sporulation were recorded.

The second method involved placing filter circles (1.25 cm diameter) on malt-yeast extract agar plates. Ethyl acetate extracts from culture samples or cyclosporin A standards in acetonitrile were placed on these filter disks, preincubated for 24 h at room temperature to allow diffusion of the cyclosporin, then seeded with *A. niger* spores and incubated as described in the first method. The diameter of the zones of inhibited growth and sporulation of *A. niger* were measured.

Figure 3 Calibration Curve of Cyclosporin Absorbance

Genuine Cyclosporin A was dissolved in HPLC grade acetonitrile. This stock solution was further diluted to give 0-1.0 µg of cyclosporin in a 10 µL injection. Duplicate injections into the HPLC system were made with each of the standard solutions. The aborbance at 214 nm was recorded by an integrator and the peak area of the Cyclosporin A absorbance was plotted against the quantity of injected compound.

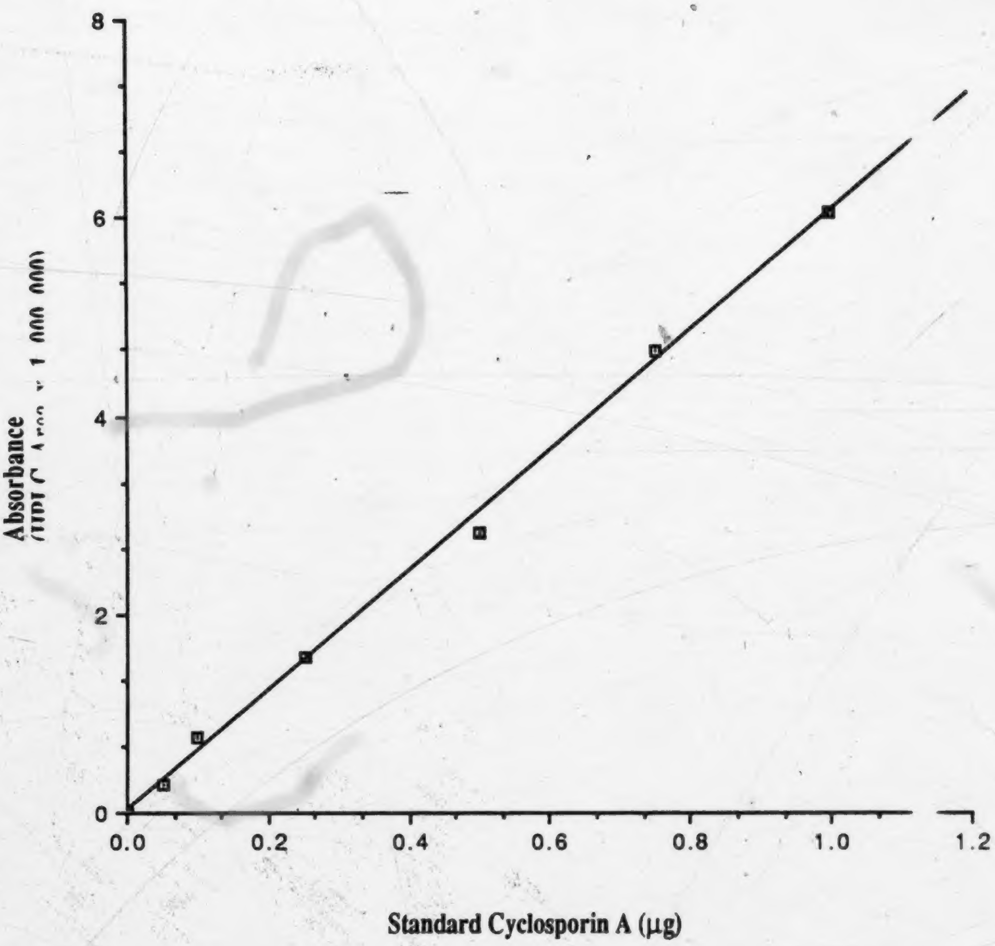
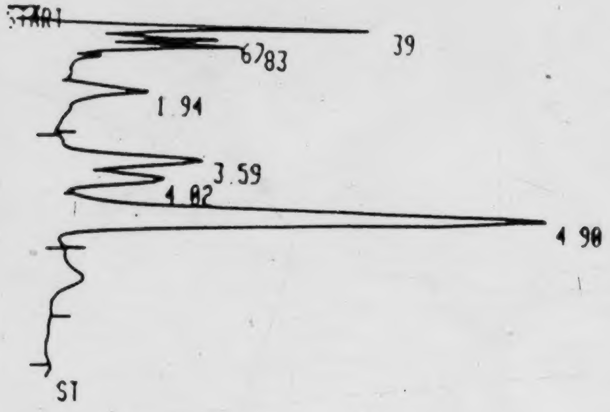

8
6
4
2
0
Absorbance
(HPLC A200 x 1,000,000)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Standard Cyclosporin A (µg)

Figure 4 Typical Chromatogram from HPLC Analysis of *T. niveum* Metabolites

A homogenate from an airlift fermenter was extracted with ethyl acetate and one mL of the organic phase was dried with forced air then the residue redissolved in 500 µL of HPLC grade acetonitrile. 10 µL of this solution was injected into the HPLC under the standard isocratic conditions and the resulting chromatogram is shown.

RT (min.)	Compound
3.59	Cyclosporin C
4.02	Cyclosporin B
4.90	Cyclosporin A



```
RUN # 6436                        JUN/07/88  14:27:35
WORKFILE ID: C
WORKFILE NAME:

AREA%
  RT           AREA TYPE     AR/HT         AREA%
  0.39       300760   BV     0.135        11.372
  0.67       164110 D VV     0.137         6.205
  0.83       205520 D VV     0.152         7.771
  1.94       255210   VB     0.375         9.650
  3.59       284150   PV     0.297        10.744
  4.02       209250   VV     0.298         7.912
  4.90      1225700   VB     0.380        46.346

TOTAL AREA=     2644700
MUL FACTOR= 1.0000E+00
```

2.6 Analytical Methods

Measurments of pH were carried out using a Fisher Accumet model 230 pH/Ion meter and Fisher (Ingold) combination probe calibrated to pH 4.0. Mycelial dry weight determinations were made by filtering 10 mL of culture homogenate through Millipore HA filter paper (0.45 µm). The filter papers were then dried overnight (minimum of 12 h) at 70°C and weighed.

Samples were centrifuged (3000 x g, 10 min) prior to sorbose and amino acid determinations and the supernatant was analyzed. Sorbose content was determined by a cysteine-sulfuric acid method described by Dische (1962). Supernatant samples (0.4 mL) containing 50-500 mg sorbose were mixed with 0.1 mL 20% L-cysteine hydrochloride monohydrate and 5 mL of 75% (v/v) sulfuric acid. The mixture was left at room temperature for 4 hours and the absorbance at 412 nm read. Amino acid content of the culture supernatant was determined by the fluorodinitrobenzene assay for free amino groups (Lowry, 1966). Samples containing 0-40 µg of vitamin assay casamino acids in 20 µL of 1% $K_2B_4O_7$ were mixed with 2 µL of FDNB reagent (130 µL of dinitrofluorobenzene in 10 mL of 100% ethanol) and heated at 60°C for 30 min. The mixture was acidified with 80 µL of 2N HCl and the absorbance read at 420 nm.

2.6.1 Production of Radiolabelled Cyclosporin

Two feeding regimes were used to produce ^{14}C-labelled cyclosporin, both of which involved adding 2.0 µCi of ^{14}C-methyl-methionine to *T. niveum* cultures. Precultures using 2% sorbose-1% casamino acid medium were inoculated with 10^8 spores of *T. niveum* UAMH 2472, incubated 165 h, homogenized and 10 mL of preculture was used to inoculate each production flask. Five production flasks (2% sorbose-1% vitamin assay casamino acid medium) were inoculated in each series. In the first method, 2.0 µCi of the labelled methionine were added to each of the five flasks at 72 h. The second method involved adding 0.33 μCi of the labelled methionine at 72, 149 , and 240 h. One flask from each set was sampled daily to determine uptake into the cells by measuring the radioactivity remaining in the supernatant. This was done by taking 2.0 mL of the culture, filtering this through a Millipore HA filter (0.45 μm), and taking three 0.2 mL samples of the filtrate. These triplicate samples were counted by liquid scintillation and averaged.

2.6.2 Liquid Scintillation Counting Methods

Radioactivity in sample supernatants was determined by liquid scintillation counting. Two mL of the appropriate culture were filtered and triplicate 0.2 mL aliquots of the supernatant were mixed with 10 mL of Aqueous Counting Solution (ACS) and counted using a Beckman LS3801 liquid scintillation counter (program 2: general ^{14}C samples). The final pooled samples were counted by adding 0.2 mL of the acetonitrile solution to 10 mL ACS then counting as above. Mycelial radioactivity was obtained by determining the dry weight of a sample and adding a known mass of dried mycelia to a counting vial. Ten mL of ACS was added and the dried mycelia allowed to settle to the bottom of the vial (to give 2π geometry for counting) and was counted as above.

2.6.3 Identification of Cyclosporins by Mass Spectrometry and HPLC Elution

Standards of cyclosporin A and D were kindly provided by Sandoz Ltd. Montreal P.Q. courtesy of Dr. G.F. Murphy. These were used to identify cyclosporins in HPLC analysis by comparison of elution times. Cyclosporins B and C had been reported as being commonly produced in *Tolypocladium* sp. cultures and indeed two peaks were observed in the culture extracts which could be cyclosporin B and C on the basis of order of elution but positive identification of eluted peaks could not be made as no standards of cyclosporins B or C were available.

In an effort to produce genuine cyclosporin B and C, five strains of *T. niveum*, UAMH 2472, 2880, 4002, 4594, and 4828 were precultured from sporulating plates then 1 mL of preculture was inoculated into each of twenty flasks for each strain. These flasks were incubated 15 days, pooled and concentrated. The concentrates were extracted overnight twice with ethyl acetate then the extracts were pooled and dried with forced air. The crude cyclosporins were taken up in chloroform and run through a silica Gel 60 chromatography column and eluted in a chloroform:methanol gradient (Dreyfuss *et al.* 1976). On the basis of HPLC analysis of the individual fractions, those fractions containing cyclosporin were pooled. Two fractions, one containing cyclosporin A alone and the other containing presumably cyclosporins B and C were collected and the latter was then run on a Sephadex LH-20 column but no further separation of the two components could be achieved. Therefore the two samples were dried and submitted to the Department of Chemistry for mass spectrometric analysis.

2.7 Fermenter Designs

Three designs of fermenter were used. The majority of work was carried out using a central draught-tube airlift design. This LH model 520 fermenter (2L operating volume, height/diameter ratio=5.5, sparge ring) with a central draught-tube (5.5 cm diameter x 36.5 cm) was equipped with air, temperature, and foaming control. Cultures were incubated at 27°C and sparged with enough air to circulate the mycelium. A number of experiments were carried out in an external loop airlift fermenter (height/diameter ratio=10, operating volume 1L, sintered glass sparger). The air supply for both was prescrubbed with glass wool and calcium chloride then passed through two sterile 0.2 µm filters. The third design was a New Brunswick Microferm stirred tank fermenter with temperature and air control. The fermenter had a height/diameter ratio of 2 and an operating volume of 10L. Aeration was set at 3L/min, the temperature maintained at 27°C, and agitation set at 200 rpm.

2.8 Immobilization of *T. niveum*

Precultures (45 h) of *T. niveum* were homogenized in a Sorvall Omnimixer at full speed for 10 s. The homogenate was immobilized in 1.5% κ-carrageen (NJAL 798, Marine Colloids Division, FMC Corporation Inc., Rockland, Maine) as previously described by Tosa (1979). Equal volumes of homogenized culture and sterile 3% (w/v) aqueous κ-carrageenan were mixed and extruded dropwise from a sterile 50 mL syringe fitted with a 20-gauge needle into 200 volumes of sterile hardening solution (0.3M KCl, 0.01 M $CaCl_2$) at 4°C. The resultant beads (2-4 mm diameter) were gently stirred for 1 h and washed by decanting with 2L of sterile distilled water.

Immobilization of spores in 2% alginate (calcium alginate, BDH Chemicals, Toronto, Ont.) was carried out as described by Deshpande (1987). *T. niveum* UAMH 2472 spores (6.5 x 10^8) from glycerol spore stocks were added to warm sterile 2% (w/v) alginate (50 mL). This mixture was extruded (as above) into 20 volumes of sterile gelling solution (2% $CaCl_2$) at 24°C, the resultant beads (2-3 mm diameter) gently stirred for 40 min., then washed with 1M NaCl.

2.9 Methods of Sorbose Sterilization

Three methods of sorbose sterilization were utilized. The first was *in situ* sterilizationwhereby the sorbose was autoclaved with the other medium components for 15min. The second method was separate autoclaving of the sorbose. This method involved autoclaving a 20% solution of sorbose of 15min. then adding the sorbose to the other autoclaved medium components. The third method was filter sterilization of the sorbose. A 20% sorbose solution was passed through a Millex GS filter (0.22 μm pore size) and the filtrate was added to the remaining autoclaved medium components.

2.10 Statistical Analysis

Data were subjected to statistical analysis by Duncan's Multiple Range test using an APL function with a MacIntosh computer. All tests were carried out at the 95% confidence level.

2.11 Chemicals

κ-Carrageenan was a gift from FMC Marine Colloids Division under the name Gelcarin CIC 789. L(-)-Sorbose, D-glucose, choline chloride, betaine, L-carnitine, and N,N-dimethylglycine were from Sigma. Casamino acids, vitamin assay casamino acids, Bacto-Casitone, Bacto-Peptone, and Tryptone were from Difco. The ^{14}C-methyl-methionine was from Amersham Corp. and was kindly supplied by Dr. P.M. Fedorak. All inorganic chemicals used were of analytical grade except those used for HPLC analyses which were of HPLC grade.

Chapter 3: Results

3.1 Initial Comparison of *Tolypocladium* and *Cylindrocarpon* Isolates for Cyclosporin Production

Nine *T. niveum* isolates were obtained from UAMH and repeated experiments were carried out to determine the highest cyclosporin-producing strains. *Cylindrocarpon lucidum* was also screened in later experiments as it had been described as producing cyclosporin on solid medium (UAMH catalogue). These growth experiments included as a control the *T. inflatum* NRRL 8044 strain (=*T. niveum*), which had been shown to produce only low levels of cyclosporin in this lab. The results from these screenings are summarized in Table 4. The final ranking of each strain is an average of the individual experiment's ranking. From these three experiments it was obvious that there could be a number of variables affecting the results. A large degree of this variability was due to the differences in the methods and sampling in each of these experiments. The first experiment involved growth of the fungus in duplicate flasks sampled after 15 days incubation, the second involved triplicate flasks of each strain (which normally only showed agreement for the low producing strains) sampled after 10 and 15 days incubation, the third experiment involved triplicate flasks of each strain with one of the three flasks sampled each day (ie. day 1, 2472 A; day 2, 2472 B; etc.). There was considerable variation between the three flasks of a particular strain. The literature had shown that 10-15 days incubation were required to exhibit the maximum cyclosporin levels (Dreyfuss *et al.* 1976: Agathos *et al.* 1986). By comparing the rankings within each screening it was possible to determine which strains produced the highest levels of cyclosporin on glucose-casamino acids medium. From these rankings, and the time course data from the third experiment, five strains of *T. niveum* were chosen for further work: UAMH 2472, 2880, 4002, 4594, and 4828 . These strains were preserved as 20% glycerol spore stocks kept at -75°C to reduce inoculum variability. It should be noted that in the first experiment,

Table 4 Cyclosporin Production by *Tolypocladium* and *Cylindrocarpon* strains

FUNGUS	Cyclosporin Production after 15 days (mg/L) (RelativeRank)			Final Ranking
	Expt. 1	Expt. 2	Expt. 3	
T. niveum UAMH 2472	125 (2)	181 (2)	75 (2)	2
T. niveum UAMH 2880	25 (10)	98[a] (4)	6 (9)	8
T. niveum UAMH 4002	54 (5)	215 (1)	127[b] (1)	1
T. niveum UAMH 4553	81 (4)	52 (6)	15 (7)	5
T. niveum UAMH 4594	126 (1)	58 (5)	25 (4)	4
T. niveum UAMH 4740	37 (7)	10 (10)	9 (8)	6
T. niveum UAMH 4828	91 (3)	101 (3)	43 (3)	3
T. niveum UAMH 4900	6 (12)	23 (8)	18 (6)	9
T. niveum UAMH 4901	30 (8)	37 (7)	22 (5)	7
T. inflatum clone II	30 (9)	4 (11)	5 (11)	11
T. inflatum clone III	44 (6)	14 (9)	5 (10)	10
T. inflatum clone IV	25 (11)	-----	-----	12
C. lucidum UAMH 4898	-----	0 (12)	0 (12)	13

[a]= one flask over 300 mg/L was not included in average
[b]= day 14 sample

Spores washed from sporulating plates of each strain were inoculated into 25 mL of 1.6% glucose medium (1% casamino acids, 0.5% KH_2PO_4, 0.5% $NaNO_3$, 0.5% NaCl, 0.5% $MgSO_4$ and trace elements) in 125 mL flasks. These precultures were incubated 7 days, then 10 mL of preculture was inoculated into duplicate (Expt. 1 or triplicate (Expt. 2 & 3) flasks containing 90 mL of medium. These production cultures were incubated 15 days then samples were withdrawn for analysis.

the *T. niveum* strains produced a brown to purple pigment which was not observed in other screenings nor in subsequent work.

Later experiments using all five of these strains showed no significant differences in the relative amount of cyclosporin produced (Table 7, Table 8, and Table 15). Therefore in the experiments that follow, a decision was made arbitrarily to use the strain UAMH 2472. However, in some fermenter experiments where only one strain was used for the experiment, *T. niveum* UAMH 4594 was used. At this point Dr. Alan Jones (Alberta Research Council) chose to work on *T. niveum* UAMH 2880 for his parallel experiments into mineral requirements of cyclosporin fermentations.

To determine if data fromn the two strains of *T. niveum* could be exchanged, several experiments compared the cyclosporin production of UAMH strains 2472 and 2880. To confirm that UAMH 2472 was the strain of choice under the conditions used, an experiment was carried out with five flasks of each strain (10^8 spores) and five flasks of UAMH 2880 (10^9 spores). The results conclusively showed that UAMH 2472 was a higher producer of cyclosporin under the conditions of the experiment. This was supported by Duncan's Multiple Range analysis of the data.

A further experiment comparing these two strains was done using equivalent spore inocula (10^8 spores) on three concentrations of sorbose, 0%, 2%, and 4% with the vitamin assay casamino acids medium (since Dr. Jones used a different sorbose concentration (4%) than this lab (2%) and technical casamino acids as the nitrogen source). The results demonstrated for both 2% and 4% sorbose UAMH 2472 produced higher levels of cyclosporin with an initial inoculum of 10^8 spores. Additionally a higher spore inoculum, 10^9 spores, did not yield higher cyclosporin production. These results (see Table 10) and their statistical analysis indicate that the UAMH 2472 strain seems best suited for these investigations into the production of cyclosporin by *T. niveum* and was used throughout most of the following experiments.

3.2 Confirmation of *T. niveum* Metabolites as Cyclosporins

3.2.1 Mass Spectroscopic Analysis of *T. niveum* Metabolites

While standards of cyclosporin A and D had been kindly provided by Sandoz the positive identification of two major classes of cyclosporins could not be made as there were no standards of these cyclosporins available. To resolve this, the following experiment was carried out to produce sufficient quantities of cyclosporins for mass spectrometry to establish the identity of two of the major peaks of a typical HPLC profile. Twenty flasks of each of the five highest cyclosporin-producing strains were grown and extracted. These extracts were separated by silica Gel 60 chromatography. Cyclosporins A, B, and C were eluted in the 99.5:0.5 fractions (chloroform:methanol gradient). A clean cyclosporin A peak was eluted in the 96:4 fraction. Subsequent chromatography using LH-20 failed to separate cyclosporin B and C. The results of mass spectrometric analysis (which included genuine cyclosporin A as a control) indicated that the fraction containing clean cyclosporin A had the same molecular weight, 1203 daltons, and the same ion fractionation pattern as the authentic cyclosporin A, 1202.6 daltons, and was therfore confirmed as cyclosporin A. The sample with the presumed cyclosporins A, B, and C showed three peaks at 1203, 1189, and 1219 daltons (Figure 5). These corresponded to cyclosporin A (1202.6), cyclosporin B or E (1188.6), and cyclosporin C (1218.6) respectively. A smaller peak was observed at 1175 daltons which was thought to be demethylated cyclosporin B or E. On the basis of order of HPLC elution it was determined that the peak at 1189 daltons could not be cyclosporin E as its position 11 L-valine is not methylated, making the molecule less hydrophobic than cyclosporin C and the cyclosporin B/E peak was eluted after the cyclosporin C (indicating its more hydrophobic nature). Also cyclosporin E has not been observed in any significant quantities in cultures. This evidence strongly suggests that the peaks observed in the HPLC analysis were cyclosporins A, B, and C.

Once it had been established that the *T. niveum* extracts contained metabolites with

Figure 5 Mass Spectrometric Analysis of *T. niveum* Metabolites

A large volume of culture homogenate was extracted with ethyl acetate in several lots and the organic phases were collected. This pooled product was separated by silica gel 60 column chromatography with a chloroform:methanol gradient (Dreyfuss *et al.* 1976). A fraction containing cyclosporins A, B, and C was dried and submitted to the Department of Chemistry for mass spectrometric analysis.

Peak Molecular Weight (daltons)	Compound
1189	Cyclosporin B
1203	Cyclosporin A
1219	Cyclosporin C

Figure 5 Mass Spectrometric Analysis of *T. niveum* Metabolites

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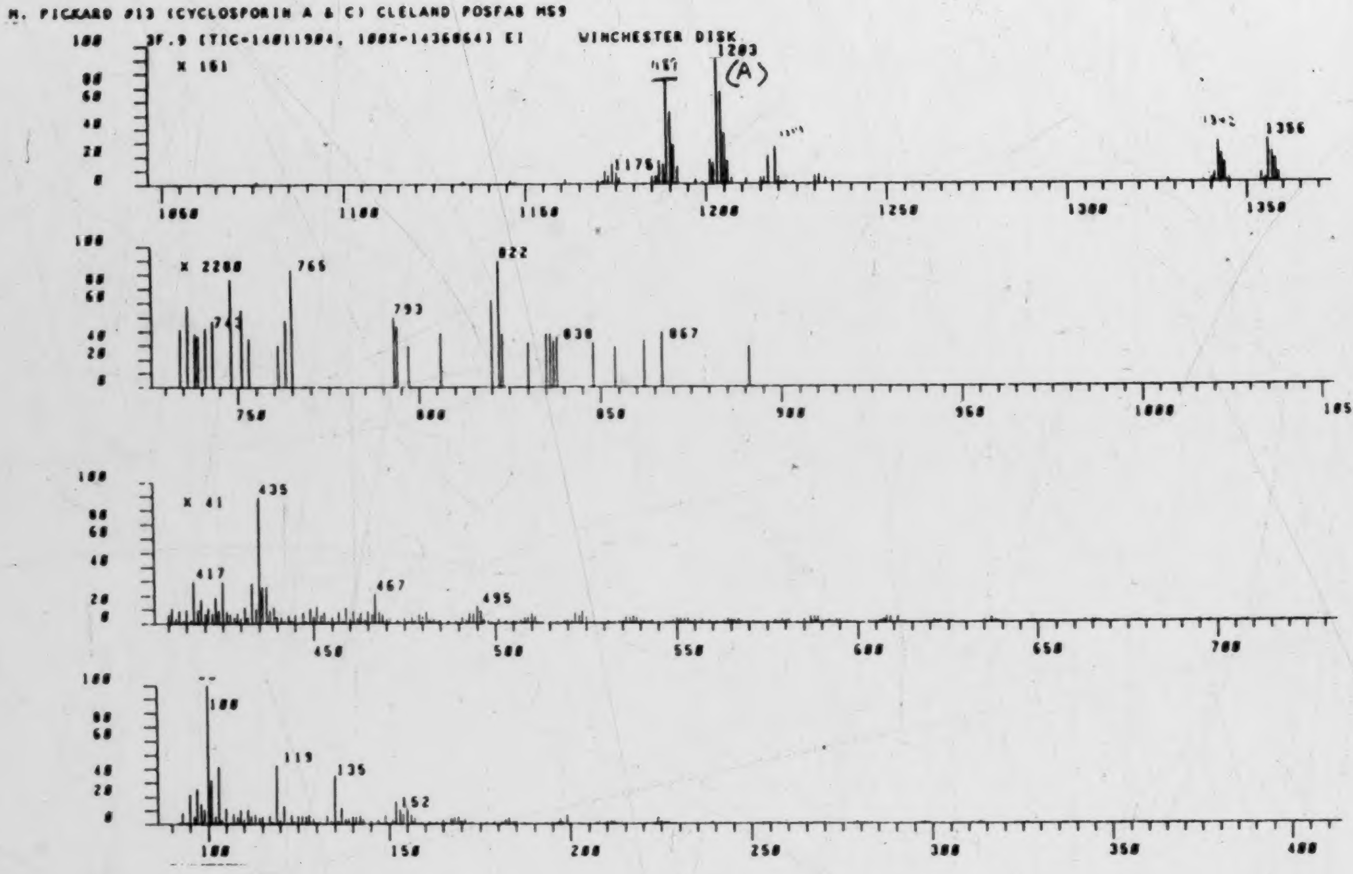

M. PICKARD #13 (CYCLOSPORIN A & C) CLELAND POSFAB MS9
3F.9 (TIC=14011904, 100%=1436064) EI
WINCHESTER DISK
X 161
1176
1203
(A)
1342
1356
1050
1100
1150
1200
1250
1300
1350
X 2200
743
765
793
822
838
867
750
800
850
900
950
1000
1050
X 41
417
435
467
495
450
500
550
600
650
700
100
119
135
152
100
150
200
250
300
350
400
100
80
60
40
20
0

the same chemical nature as genuine cyclosporins, the biological activity of these metabolites was examined. The method of detecting biological activity was in the form of a bioassay which could hopefully also be used to quantitate cyclosporin produced.

3.2.2 Biological Activity of *T. niveum* Metabolites

A. niger sporulation has been shown to be inhibited in the presence of cyclosporins, and this biological activity was exploited in a bioassay. Two types bioassays were devised which involved measuring the zones of inhibited sporulation. One method tested cyclosporin production of *T. niveum* and *C. lucidum* growing on solid agar while the second method tested the cyclosporin content of ethyl acetate extracts from *Tolypocladium* and *Cylindrocarpon* submerged cultures.

Method one, the whole-cell agar plug method, showed little correlation between a particular strain's activity and its previous ability to produce cyclosporin. For the *T. inflatum (=T. niveum)* strains tested there was a large zone of inhibition (70 mm diameter) and a high degree of killing but several of these strains had been shown to produce only low levels of cyclosporin. Conversely high producers such as *T. niveum* UAMH 4594 showed smaller zones of inhibition (15-20 mm diameter) and no killing. It can be suggested from these results that inhibition might be due to another factor produced by the fungi, that the incubation period was insufficient for the complete diffusion of cyclosporin, that the high producing strains only produce low levels of cyclosporin on solid medium, or that the cyclosporin was still associated with the mycelium and did not diffuse into the agar.

Method two determined the cyclosporin content of ethyl acetate extracts from 15 day submerged cultures to by inhibiting *A. niger* sporulation or growth. The results obtained from the bioassay were compared to HPLC analysis of the ethyl acetate extracts. A calibration curve constructed from the cyclosporin A standards showed no inhibition until 3 µg cyclosporin A was placed on the disk. Samples from the five strains determined to be high producers of cyclosporin also caused the largest zones of inhibition. These strains of *T. niveum* (UAMH 2472, 2880, 4002, 4594, and 4828) had zones of inhibition ranging from 17-30 mm. The cyclosporin values from the bioassay standard curve correlated well with the HPLC analyses of these extracts for the linear portion of the standard curve. A minimum of 39 mg/L (by HPLC analysis) cyclosporin concentration (3.9 µg cyclosporin on the filter disk) was required to show any inhibition of *A. niger* sporulation. This correlated well with the minimum 3 µg standard cyclosporin A which caused inhibition in the standard curve and there was reasonable agreement between duplicate samples (±15%).

These results indicated that the second bioassay method was superior to the first. The results of the former were comparable to those from HPLC analysis as a measure of the metabolite production in submerged culture rather than on solid medium. A further advantage to the second method was that the standard curve was much more linear and the sample handling was more convenient. The problem with both of these methods involved the reproducibility between samples and the seeding of the *A. niger* lawn. Simply tapping down *A. niger* spores from a sporulating plate did not give identical lawns of *A. niger* growth on the test plates. A better method of inoculating the test plates with *A. niger* must be devised. Another problem was the difficulty in handling sporulating *A. niger* plates. These spores are very easily spread and represent a serious threat to laboratory sterility if not handled properly. The second method has the potential to be exploited as a simple, reproducible method for detecting relatively high levels of cyclosporin (over 39 mg/L) in ethyl acetate extracts of cultures. However for the initial work involving low producing strains and comparative work for medium and methodology development, HPLC analysis was used. An improved bioassay would be very useful for the screening of large numbers of mutagenized clones of *T. niveum* for high levels of cyclosporin production.

3.3 Inoculum Development

One of the most variable procedures in the reports of cyclosporin fermentations is the preculture preparation with which the production medium is inoculated. Preculturing is necessary because of the need to generate biomass for the inoculation of the production medium as cyclosporin is, at least initially, intracellular and production is related to biomass. A number of preculture media have been used, the majority of which have had a complex composition such as malt extract or yeast extract. The size of the preculture inoculum transferred into the production medium was also variable; the source or maintenance of the initial spores had not been well defined nor had the age of the preculture prior to inoculation of the production medium. These factors, and other details in the preculture preparation and the production medium inoculum, were investigated in order to determine the methods which yield the highest reproducible levels of cyclosporin. However as cyclosporin production by most of these strains of *Tolypocladium* has not been reported, it cannot be assumed that the conditions for *T. inflatum* NRRL 8044 (=*T. niveum*)will be ideal. Also there are critical points involving reproducibility which are not addressed in the literature which must be investigated .

3.3.1 Determination of Optimum Conditions for Cyclosporin Production

The thrust of the initial work in cyclosporin studies was to eliminate any sources of variability. One potential source of variability in the growth of the culture and the production of cyclosporin, is the spore inoculum. To standardize this, 20% glycerol spore stocks of the five previously described UAMH strains were prepared and stored at -75°C. These spore stocks could be used immediately and allowed the inoculation of a precise number of viable spores to a culture. This method had less variability than inoculating spores washed from a plate from which the number and age of theviable spores could not readily be determined.

An experiment was carried out to determine the best spore concentration for preculture as well as the optimum volume for inoculum of the production culture. A number of preculture flasks were inoculated with spore loads ranging from 2.6 x 10^2 spores/mL medium to 1.95 x 10^4 spores/mL medium (Agathos *et al.* 1986). The medium used for both the precultures and production flasks was 1.6% sorbose-1% casamino acids medium. The final cyclosporin production was poor, less than 50 mg/L, except in one flask which produced 103 mg/L. The results indicated that some factor of the fermentation was missing. As high levels of cyclosporin (<130 mg/L) had been produced previously using similar conditions, the spore load must have been too low. The only flask which produced over 100 mg/L had a high density of spores added to the preculture (3.25 x 10^5 spores in 25 mL).

It was determined in later work that consistently high levels of cyclosporin production required >10^6 spores as the preculture inoculum in 100 mL of SVA medium, as shown in Figure 8.

3.3.2 Preculture Media and Inoculum Preparation

In our work, precultures were always grown in a semi-defined media such as the production media described by Foster *et al.* (1983) or by Agathos *et al* . (1986). All previous workers had used rich non-defined media for their precultures. To determine which type of preculture was best for cyclosporin production by *T. niveum* UAMH 2472, the following experiment was carried out. Two groups of precultures were prepared, one using malt-yeast extract medium (2% malt extract, 0.4% yeast extract) and the other using SVA medium. 100 mL of medium was inoculated with 10^8 spores and incubated 72 h. Half of each preculture was washed by sedimentation and resuspension in 1% KH_2PO_4-0.5% KCl (sorbose medium salts), then flasks of sorbose production medium were inoculated with various concentrations of washed and unwashed precultures. These production flasks were incubated 15 days. The results (Figure 6) show clearly that preculturing in sorbose medium yields higher levels of cyclosporin than a malt-yeast extract medium. The final biomass of both sets of production flasks was virtually identical for all inoculum sizes though the biomass of the malt-yeast extract precultures was almost twice that of the sorbose precultures (8.0 g/L unwashed versus 4.3 g/L unwashed). Washing the preculture had little effect on the final cyclosporin production even though the biomass of the washed preculture inocula was 40% lower than the unwashed precultures for both malt-yeast and sorbose precultures as determined by mycelial dry weight. Statistically there was no difference between the cyclosporin yield of different inoculum sizes. Therefore unless carryover of nutrients or minerals from the preculture was a concern, precultures were not washed unless indicated. As the size of the production culture inoculum had no effect on final production, the 10% inoculum was used in all later experiments unless indicated.

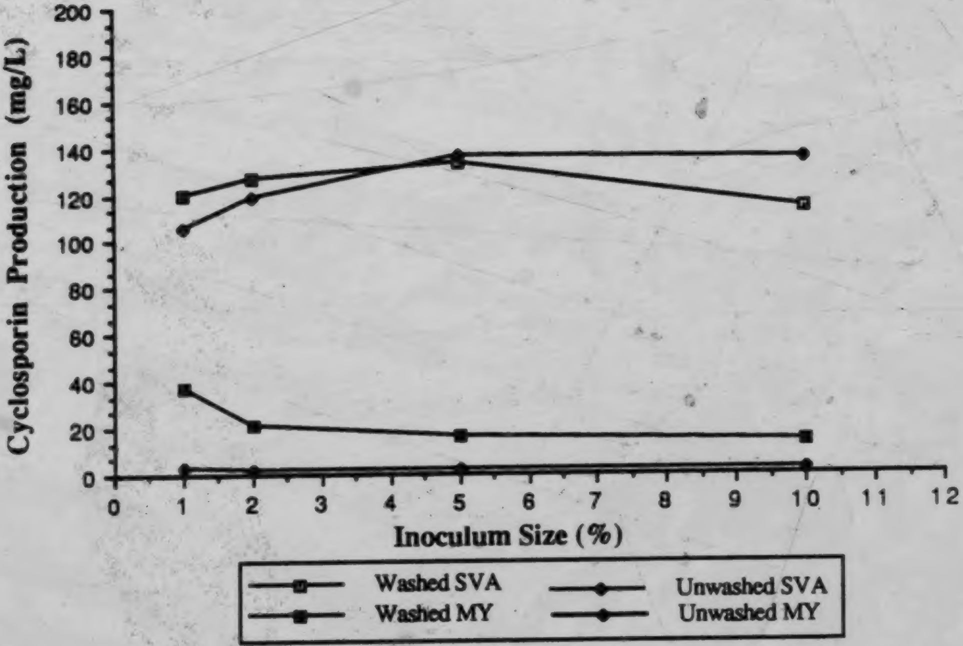
3.3.3 Optimization of the Inoculum for Production Culture

The state of the preculture when it is inoculated into the production medium is of importance if the fermentation is to be optimized. The fungus in preculture must be in a state that is most conducive to cyclosporin production. The following experiments were carried out to optimize the precultures for cyclosporin production.

One of the variables which was altered was the age of the preculture. Precultures were prepared and at time periods inoculations from these precultures were made into triplicate flasks of production medium. The results (Figure 7) indicate that there was no significant difference in the final cyclosporin production among any of the inocula grown for different time periods and was confirmed by Duncan's Multiple Range analysis. This indicated that if the inoculum to the production medium had a sufficient biomass (as all those tested had) the final cyclosporin production over 15 days would not be altered. As all

Figure 6 Comparison of Preculture Media and Inoculum Washing for Cyclosporin Production

Two preculture series, malt-yeast extract medium (MY) and SVA medium (SVA) were prepared and inoculated with 10^8 spores of *T. niveum* UAMH 2472 from glycerol spore stocks and incubated 72 h. Each preculture was then split and half of each was washed by repeated sedimentation and resuspension with a 1% KH_2PO_4-0.5% KCl solution. The precultures, both washed and unwashed, were then inoculated into SVA medium at different concentrations. These production cultures (duplicate cultures for each treatment) were incubated 15 days, homogenized, and analyzed.


Cyclosporin Production (mg/L)
0
20
40
60
80
100
120
140
160
180
200
0
1
2
3
4
5
6
7
8
9
10
11
12
Inoculum Size (%)
Washed SVA
Washed MY
Unwashed SVA
Unwashed MY

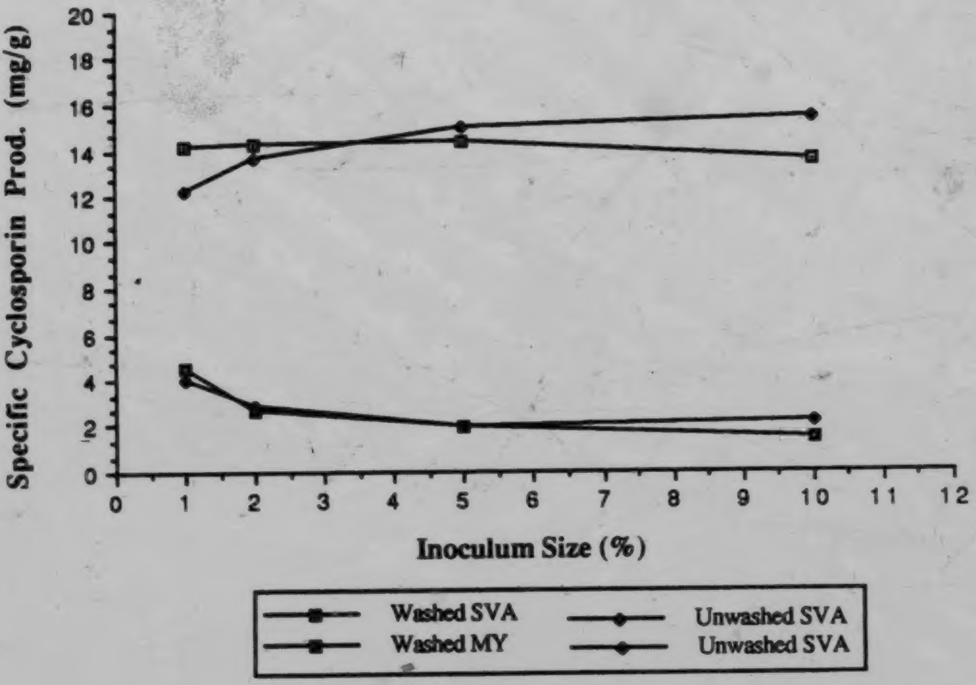
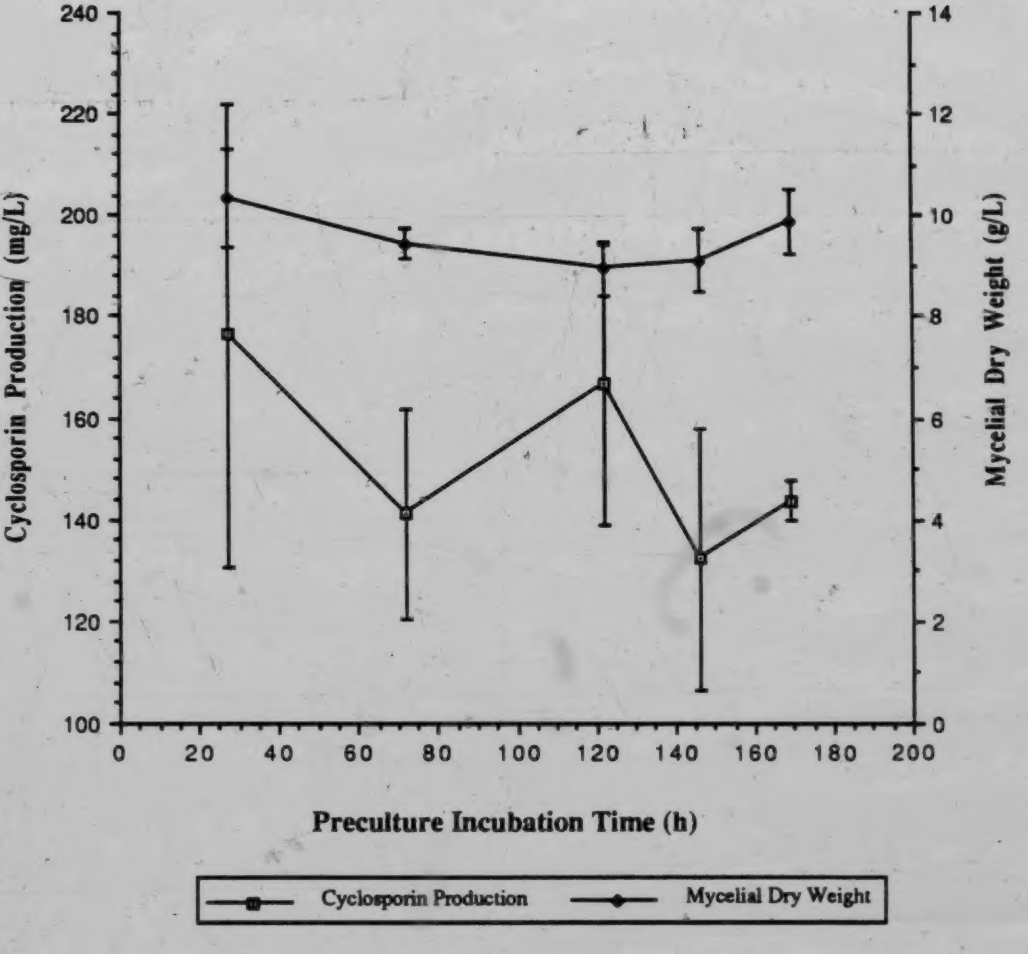

Specific Cyclosporin Prod. (mg/g)
0
2
4
6
8
10
12
14
16
18
20
0
1
2
3
4
5
6
7
8
9
10
11
12
Inoculum Size (%)
Washed SVA
Washed MY
Unwashed SVA
Unwashed SVA

Figure 7 Effect of Preculture Age on Cyclosporin Production

Preculture flasks containing 100 mL of SVA medium were inoculated with 10^8 spores of *T. niveum* UAMH 2472 from glycerol stocks and were incubated for different times. 10 mL of each preculture was inoculated into triplicate flasks containing 100mL of SVA medium and these production cultures were incubated 15 days. These cultures were homogenized and analyzed. The error bars represent one standard deviation from the mean.


Cyclosporin Production (mg/L)
240
220
200
180
160
140
120
100
Mycelial Dry Weight (g/L)
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
160
180
200
Preculture Incubation Time (h)
Cyclosporin Production
Mycelial Dry Weight

the preculture inocula yielded statistically equal levels of cyclosporin, 96 h was chosen as the preculture time to ensure there was growth in the preculture but not to needlessly extend the preculture time (inoculum could be grown over the weekend).

The quantity of biomass of the precultures was also investigated to determine any effect on final cyclosporin production. Inoculum biomass was related to the volume of the preculture added. As was observed in Figure 6, the volume of inoculum had no significant effect on final levels of cyclosporin production. To further investigate this, two experiments were carried out both using 96 h precultures. The first experiment examined the final cyclosporin production of flasks from varying inoculum sizes from 6.15 to 123 mg. However Duncan's Multiple Range analysis of the results (Table 5) showed that only the highest and lowest inoculum biomasses, 123 and 6.15 mg respectively differed in final production of cyclosporin. Also the control level, 61.5 mg, produced much lower levels of cyclosporin than previously observed. The experiment was repeated excluding the highest inoculum size. This experiment again showed no significant difference between production culture inoculum biomass and cyclosporin production. Therefore until contraindicated, a 10% inoculum of preculture was used as the inoculum.

3.3.4 Effect of Spore Source on Cyclosporin Production

The question of comparing cyclosporin production from fresh spores with that from the frozen (-75°C) glycerol spore stocks had been raised. Preculture flasks and malt-yeast extract plates were inoculated with 10^8 spores from the glycerol spore stocks and incubated 7 days. At this time the plates were washed with distilled water and two sets of preculture flasks were inoculated from the resulting spore suspension, one set received 1.8×10^6 spores/flask and the other received 2.7×10^6 spores/flask. These flasks were also incubated for 7 days. Five flasks of SVA medium were inoculated from each preculture (10% inoculum) and incubated 15 days. The results indicated that the fresh spores at 1.8 x

Table 5 Effect of Inoculum Biomass on *T. niveum*

Inoculum Biomass (mg)	Mycelial Dry Weight (g/L) (± S.D.)		Cyclosporin Production (mg/L) (± S.D.)		Specific Cyclosporin Production (mg/g) (± S.D.)	
6.15	8.6	(0.0)	109	(3)	12.6	(0.4)
12.3	8.8	(0.0)	99	(11)	11.3	(1.2)
30.8	8.1	(0.2)	86	(5)	10.6	(0.5)
61.5	8.9	(0.0)	88	(4)	9.8	(0.4)
92.2	8.1	(0.1)	99	(16)	12.2	(1.8)
123	8.6		81		9.4	
8.6	9.1	(0.4)	85	(5)	9.4	(0.6)
17.2	9.3	(0.2)	92	(18)	9.9	(1.8)
43	9.3	(0.5)	86	(9)	9.3	(1.3)
64.5	9.1	(0.2)	98	(3)	10.8	(0.2)
86	8.9	(0.5)	78	(20)	8.8	(2.5)

These results are from two experiments. The protocol for both involved the inoculation of 10^8 spores of *T. niveum* UAMH 2472 from glycerol spore stocks into 100 mL of SVA medium. These precultures were incubated 96 h then the biomass in the precultures was determined. Different sizes of inoculum were added to production flasks containing 100 mL of SVA medium. These were incubated 15 days then made up to original volume with distilled water, homogenized, and analyzed. All the cultures were carried out in triplicate except the 92.2 mg series (duplicate) and the 123 mg series (single flask).

10^6 level yielded highest cyclosporin production of 202±21 mg/L. The glycerol spore stocks (10^8 spores) and the fresh spores at 2.7 x 10^6 level yielded statistically equivalent cyclosporin production of 162±10 mg/L and 158±31 mg/L respectively. The flasks all produced high levels of cyclosporin both volumetrically (mg/L) and specifically (mg/g dry weight mycelium). The fresh spores appeared to be equivalent if not superior to the glycerol spore stocks, though there was slightly more variability in the fresh spores' cyclosporin production. For reproducibility and convenience the use of the glycerol spore stocks was continued.

3.3.5 Spore Inoculum Effects on *T. niveum* Morphology

The morphology of submerged fungal cultures has been shown to be affected by the size of the inoculum. Inocula of low spore concentrations yield a pelletized morphology while higher levels of inocula yield a more filamentous morphology (Metz and Kossen, 1977). This had been observed in *T. niveum* cultures both in shake flask and fermenter cultures. To determine the spore inoculum necessary to obtain pellet morphology as well as to compare the production of pelletized *T. niveum* with filamentous mycelia the following experiment was carried out.

Glycerol spore stocks were diluted with distilled water to various concentrations and inoculated into precultures. The precultures were incubated 14 days to allow complete growth in all flasks and were inoculated (at 10%) into quadruplicate production flasks containing 100 mL of SVA medium. The precultures were then sampled for cyclosporin production. The production flasks were incubated 15 days. The results in Table 6 show that precultures receiving 10^4 spores or less, grew in pellets while those receiving higher numbers of spores grew as filamentous mycelia. Production in all the precultures was very high, up to 230 mg/L (note that these precultures were 15-20% concentrated due to evaporation during incubation) but generally was lower for the lower spore inocula with

Table 6 Effect of Inoculum Size on Preculture Morphology and Cyclosporin Production

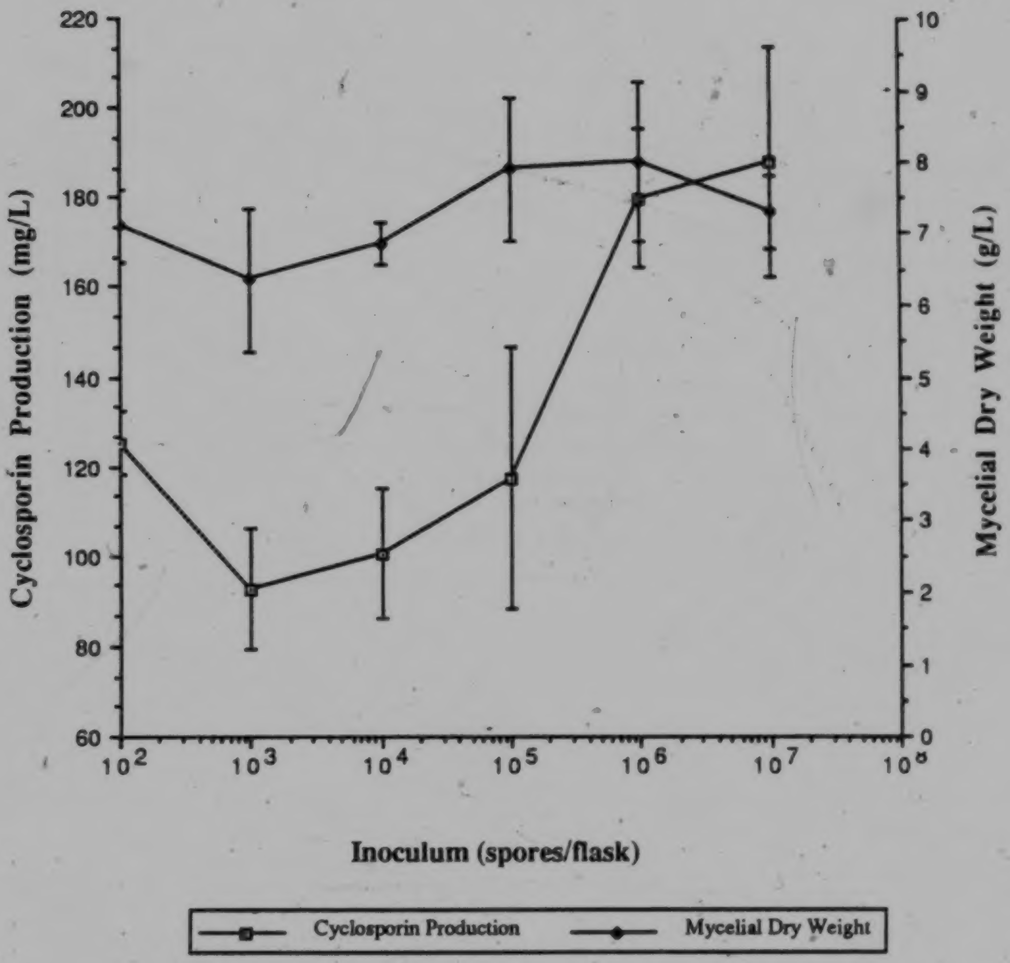
Spore Inoculum (Spores/flask)	Mycelial Dry Weight (g/L) (±S.D.)	Cyclosporin Production (mg/L) (± S.D.)	Mycelial Morphology
10^7	11.2 (0.1)	229 (28)	Filamentous
10^6	8.9 (0.2)	207 (51)	Filamentous
10^5	7.6 (0.1)	202 (21)	Filamentous
10^4	8.8 (1.1)	134 (44)	Pellet
10^3	5.6 (0.4)	84 (18)	Pellet
10^2	7.9 (1.5)	186 (16)	Pellet

These results are the cyclosporin production, mycelial dry weight, and morphology of the precultures which were prepared in the following manner. Glycerol spore stocks of *T. niveum* UAMH 2472 were serially diluted with phosphate buffer. The diluted spore suspensions were inoculated into duplicate flasks containing 100 mL of SVA medium. These flasks were incubated 14 days until all flasks demonstrated good mycelial growth. At this point the production cultures were inoculated and the precultures analyzed for pH, mycelial dry weight, and cyclosporin content.

pelletized growth. The production cultures had the same morphology as their precultures, if pellets were inoculated the culture had a pellet morphology. All the production flasks contained equivalent biomass whether growing as filamentous mycelium or as pellets. The mycelial dry weights for these flasks were between 6 and 8 g/L. Cyclosporin production reflected the general trend observed in the precultures, filamentous cultures produced higher levels of cyclosporin than pelletized cultures. These results are shown in Figure 8. The increase in cyclosporin production as the spore inoculum increased is quite marked for the 10^6 and 10^7 spore inocula and leveled off at 180 mg/L cyclosporins. It appears that there was a critical spore concentration below which pellets formed. The formation of pellets may be the result of electrostatic attraction (Jones *et al.* 1988) or other attractive forces such as hydrophobic interactions. All pellets observed in shake flasks and fermenters appear to be clumps of germinated spores which are attracted to each other while germinating and consequently grow as an aggregate (Figure 9). The higher spore load in the filamentous morphology also showed clumping and aggregation of the spores but the high number of spores inoculated resulted in many very small aggregates rather than fewer large pellets. The high cyclosporin levels observed in the 10^2 spores cultures might have been due to the fact there were so few spores that practically each spore developed into a pellet. Therefore the total surface area of growing mycelia of the pellets was greater than the less numerous larger pellets observed in flasks with higher spore inocula. It is obvious that there was simply too little mycelia transferred from the preculture to the production culture to allow for sufficient time to permit enough vegetative growth for cyclosporin production. All the production cultures produced statistically equivalent mycelial dry weights (see Figure 8). From these results it can be concluded that the highest cyclosporin levels were derived from filamentous cultures. However if pelletized mycelia were required (for a fermenter), pellets could be formed which produced reasonably high cyclosporin levels.

Figure 8 Effect of Preculture Inoculum Size on Cyclosporin Production

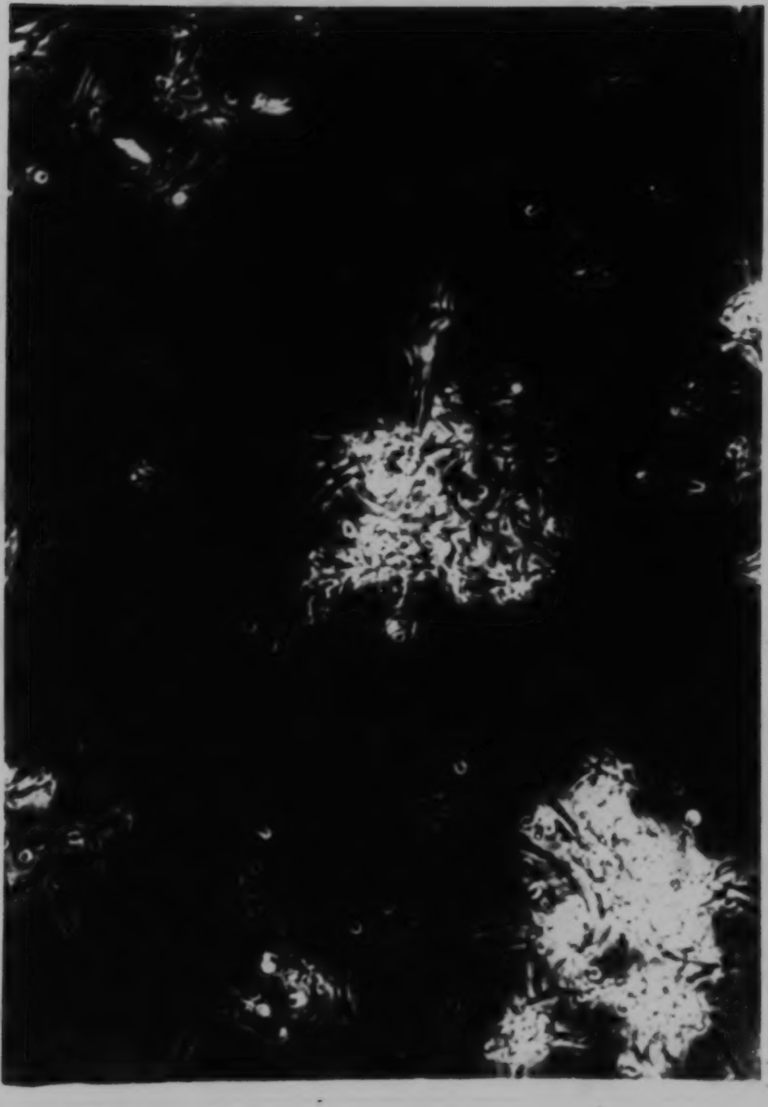
Precultures flasks containing 100 mL of SVA medium were inoculated with different concentrations of *T. niveum* UAMH 2472 spores from glycerol spore stocks. These precultures were incubated 14 days to ensure complete growth in each flasks then 10 mL of preculture was inoculated into each of quadruplicate flasks of SVA medium and incubated 15 days. These production cultures were then homogenized and analyzed.



Cyclosporin Production (mg/L)
220
200
180
160
140
120
100
80
60
Mycelial Dry Weight (g/L)
10
9
8
7
6
5
4
3
2
1
0
10^2
10^3
10^4
10^5
10^6
10^7
10^8
Inoculum (spores/flask)
Cyclosporin Production
Mycelial Dry Weight

Figure 9 Aggregation of Germinating *T. niveum* Spores

Spores from glycerol stocks were inoculated into SVA medium and incubated for 48 h. An aliquot was removed and observed under a phase contrast microscope by an oil immersion lens. Magnification of the photomicrograph is 2000x.



3.4 Medium Development

Once the fungal strain had been chosen and the basic methods established, modification of the growth medium became the primary method of improving cyclosporin production by the fungus and studying its nutritional requirements. The three major parts of any fungal growth medium are the carbon/energy source, nitrogen source, and salts. These three components should be optimized in order to obtain the highest reproducible cyclosporin production possible using a single strain of *T. niveum*.

3.4.1 Carbon Source Optimization

A great number of carbon sources had been shown to be suitable for cyclosporin production by *Tolypocladium* sp.(Agathos *et al.* 1986). The best of these carbon sources, plus glucose (Foster *et al.* 1983) were investigated to find the carbon source which yielded the highest levels of cyclosporin.

3.4.1.1 Determination of Optimum Carbon Source

An experiment was carried out to compare cyclosporin production of *T. niveum* using two growth media, 1.6% sorbose medium (Agathos *et al.* 1986) and 1.6% glucose medium (Foster *et al.* 1983). These two media were tested using four of the five highest cyclosporin producing strains of *T. niveum*. To negate any possible effects of the other medium components, the carbon sources were also used in the other medium (ie. sorbose was also used to replace glucose in the glucose medium and vice-versa). The results are shown in Table 7. Analysis by Duncan's Multiple Range test indicates that sorbose is the better carbon source. Sorbose in either medium yields statistically equivalent cyclosporin levels which are superior to those from glucose media. The cyclosporin production from the Agathos' sorbose medium, while statistically equivalent to the Foster's sorbose medium, had less variation and better reproducibility between strains and duplicates.

Table 7 Cyclosporin Production on Different Media

T. niveum Strain	Carbon Source	Medium	Cyclosporin Production (mg/L) (±S.D.)
2472	Sorbose	Agathos'	154 (6)
2472	Glucose	Agathos'	90 (11)
2472	Sorbose	Foster's	88 (8)
2472	Glucose	Foster's	145 (35)
2880	Sorbose	Agathos'	166 (9)
2880	Glucose	Agathos'	50 (0)
2880	Sorbose	Foster's	158 (39)
2880	Glucose	Foster's	61 (6)
4002	Sorbose	Agathos'	188 (16)
4002	Glucose	Agathos'	71 (16)
4002	Sorbose	Foster's	120 (34)
4002	Glucose	Foster's	116 (64)
4828	Sorbose	Agathos'	144 (11)
4828	Glucose	Agathos'	97 (37)
4828	Sorbose	Foster's	147 (8)
4828	Glucose	Foster's	31 (8)

Spores from sporulating plates of each strain were washed with sterile distilled water and inoculated into 125 mL flasks containing 25 mL of the appropriate medium. The carbon sources were added at 1.6% concentration. Foster's medium was 1% casamino acids, 0.5% KH_2PO_4, 0.5% $NaNO_3$, 0.5% NaCl, 0.05% $MgSO_4$, and trace elements. Agathos' medium was 1% casamino acids, 0.5% KH_2PO_4, and 0.25% KCl. These precultures were incubated for 7 days, then 10 mL of preculture was inoculated into flasks containing the 90 mL of the production medium. These duplicate production cultures were incubated 15 days, homogenized, and the cyclosporin content determined.

To confirm these results, another experiment was carried out to find the best carbon source for cyclosporin and the optimum concentration of that carbon source. The five highest producing strains of *T. niveum* were again used with five carbon sources, 2% and 3% sorbose, 2% and 3% fructose, and 3% sucrose. The fungi grew well on all five carbon sources and produced cyclosporin. However the cyclosporin production for all flasks was very low, less than 50 mg/L. The reason for the low cyclosporin levels was not immediately obvious as the cultures had been treated as in previous experiments. Although it was difficult to draw any conclusions from such data, statistical analysis showed 3% sucrose to be an inferior carbon source to the sorbose or fructose. These two carbon sources were equivalent for cyclosporin production at both 2% and 3% concentrations.

Further studies on the carbon source included a comparison of 2% sorbose, 2% fructose, 1% maltose, and a negative control. The experiment was carried out using only *T. niveum* UAMH 2472 at a low preculture inoculum level. The final cyclosporin production values, shown in Table 8, were again low, 65-81 mg/L cyclosporins. However the negative control had high cyclosporin levels, 104 mg/L. Why the flasks with no added carbon source should have produced such relatively high cyclosporin levels was not determined and in repeated experiments this observation was not seen again. Duncan's Multiple Range analysis showed all the carbon sources to be statistically equivalent.

Although sorbose had been used throughout most of the experiments it had not been demonstrated that sorbose was the optimum carbon source. The preceding experiments have not shown sorbose to be superior to fructose, which is less than one-quarter the cost. To resolve this another experiment was carried out comparing 2% sorbose, 2% fructose, and 2% glucose with the UAMH 2472 strain of *T. niveum*. The results (Table 8) show that the five cultures containing sorbose produced a mean of 135±23 mg/L total cyclosporins which was statistically higher than the fructose and glucose flasks which produced 100 mg/L and 74 mg/L respectively. This experiment demonstrated that

Table 8 Cyclosporin Production on Different Carbon Sources

Carbon Source	Final pH	Mycelial Dry Weight (g/L) (± S.D.)	Cyclosporin Prod. (mg/L) (± S.D.)	Specific Cyclosporin Production (mg/g) (± S.D.)
2% Sorbose	5.05	2.1	81 (11)	39
2% Fructose	5.85	1.6	65 (37)	39
1% Maltose	5.95	2.4	78 (25)	33
2% Sorbose	4.34	8.7 (0.4)	135 (23)	16 (3)
2% Fructose	4.06	7.8 (1.7)	100 (17)	13 (3)
2% Glucose	4.02	8.7 (0.3)	73 (16)	8 (2)

These results represent two experiments comparing cyclosporin production between carbon sources. In both experiments spores from glycerol stocks of *T. niveum* UAMH 2472 were inoculated into precultures and incubated. These precultures were then inoculated into production flasks and incubated 15 days. After incubation the contents of the flasks were analyzed. In the first experiment (rows 1-3), the preculture was inoculated with 3.25×10^5 spores and incubated 185 h whereas in the second experiment (rows 4-6), 10^8 spores were inoculated and incubation was for 72 h. The production medium in the first case was 2% sorbose-1% casamino acids medium and the experiment was carried out with quadruplicate flasks while the second was SVA medium and was done in quintuplicate After incubation, the cultures in the second experiment were made up to volume with distilled water and homogenized before analysis.

sorbose was a better carbon source for cyclosporin production. However fructose was also a viable carbon source and may have potential use in light of its lower cost.

3.4.1.2 Sorbose Preparation

While it has been determined in one experiment that sorbose was the best carbon source for cyclosporin production, autoclaving the sorbose medium turned the medium a golden-brown colour and produced an odor similar to burned or cooked sugar. This colour change is likely due to caramelization of the sorbose. Whether this caramelization had any effect on the carbon source was unknown and therefore was investigated.

Two experiments were carried out involving three methods of sorbose preparation and sterilization. These were: separate filter sterilization, separate autoclaving, and autoclaving with the medium *in situ*. The first experiment was carried out in triplicate and failed to show any significant difference between the three methods: the mean for *in situ* autoclaving was 42% higher than either the autoclaved or filter-sterilized methods but there was great variability between replicate flasks. The experiment was repeated in quintuplicate and the sorbose content from each flask was determined before inoculation of the production culture. The results (Table 9) indicated that *in situ* autoclaving yielded higher cyclosporin production values than either filter-sterilized or separately autoclaved sorbose. Also the fungal growth in the *in situ* flasks was heavier than in the other two methods. The results of the sorbose assay were somewhat puzzling as the chemical assay for sorbose content of the *in situ* autoclaved flasks (9 g/L) revealed only one-half that in the other two (17 g/L) but the availability of sorbose as a carbon source was apparently not affected The most likely explanation is that the sorbose and some other medium component were forming a complex in such a manner that the sorbose was not detected by the assay. The most likely candidate was the vitamin assay casamino acids which might form a Maillard product upon heating of this nitrogen source with a sugar (Bannerjee *et al*. 1979) and in

Table 9 Cyclosporin Production for Three Methods of Sorbose Preparation

Sterilization Method	Sorbose Content (g/L) (± S.D.)	Final pH	Cyclosporin Prod.(mg/L) (± S.D.)
In situ	9 (0.6)	5.6	137 (17)
Filter-Sterilized	18 (0.2)	5.4	109 (13)
Autoclaved	18 (0.3)	5.5	113 (15)

Precultures were started with 10^8 spores of *T. niveum* UAMH 2472 in 100 mL of SVA medium. These precultures were incubated 72 h. 10% inoculations were made into quintuplet flasks containing the following medium; 1% vitamin assay casamino acids, 1% KH_2PO_4, and 0.5% KCl. The sorbose was either autoclaved *in situ* with the medium, autoclaved separately then added to the medium, or filter-sterilized through 0.22 µm Millex GS filter and added to the medium, all at a 2% concentration. Sorbose content was determined by chemical analysis prior to inoculation. These production cultures were incubated for 15 days, made up to volume with distilled water, homogenized, and analyzed.

this form (a Maillard product) the sorbose was not detected. The complex cannot form in the other two methods because the sorbose and nitrogen source are never in contact during autoclaving. Therefore the method of sorbose preparation that yields the highest cyclosporin production and fungal growth was *in situ* autoclaving with the whole medium.

A further experiment was carried out to define the sorbose concentration which was better suited for cyclosporin production. This experiment compared cyclosporin production at two concentrations, 2%, and 4%. The results (Table 10) indicated that the two sorbose levels produced statistically different cyclosporin production and that 2% was the optimum concentration of sorbose. This confirmed the use of 2% sorbose through out the project.

3.4.1.3 Growth of *Tolypocladium niveum* on Tetradecane

One of the strains of *T. niveum* , UAMH 4002, was originally isolated from oil-soaked Arctic soils and shown to utilize aliphatic hydrocarbons as the carbon source for growth (Davies and Westlake, 1979). To determine if all the *T. niveum* strains could grow on complex hydrocarbons as carbon sources an experiment was carried out to determine the if the top five cyclosporin producing strains of *T. niveum* could in the presence of tetradecane and if any cyclosporin would be produced during this growth. The results indicated that all five strains grew on the tetradecane-casamino acids medium and produced low quantities of cyclosporin, 2-10 mg/L. However the amount of this growth that was due to the tetradecane cannot be determined unless the experiment was repeated using only tetradecane and inorganic nitrogen in the medium. This production might be simply due to growth on the casamino acids.

Table 10 Cyclosporin Production of Two Strains of *T. niveum* from Two Inoculum Sizes

T.niveum Strain	Spore Inoculum	Sorbose Conc. (%)	Mycelial Dry Weight (g/L) (± S.D.)		Cyclosporin Prod.(mg/L) (± S.D.)		Specific Cyclosporin Prod. (mg/g)
2472	10^8	2.0	9	(0.1)	133	(14)	14.7
2880	10^8	2.0	9	(0.8)	98	(14)	10.5
2880	10^9	2.0	10	(0.6)	13	(7)	1.3
2472	10^8	0	2	(0.3)	17	(5)	8.5
2472	10^8	2.0	9	(1.4)	133	(14)	14.8
2472	10^8	4.0	12	(0.4)	102	(7)	8.5
2880	10^8	0	3	(0.1)	13	(1)	4.3
2880	10^8	2.0	9	(0.8)	98	(14)	10.9
2880	10^8	4.0	11	(1.1)	75	(45)	6.8

These results are from two experiments. The first experiment (rows 1-3) involved inoculating 100 mL of SVA medium with either 10^8 or 10^9 spores from glycerol spore stocks of each strain. The precultures were incubated 7 days, then 10 mL of each preculture was inoculated into five flasks containing 100 mL of SVA medium. These flaskss were incubated 15 days then harvested. The cultures were made up to original volume (110 mL), homogenized and analyzed for pH, mycelial dry weight, and cyclosporin content. The second experiment (rows 4-9) involved the same procedure as described except the precultures were only incubated 72 h rather than 7 days.

3.4.2 Nitrogen Source Optimization

A great many nitrogen sources have been used in cyclosporin fermentations. The most common of these are the caseinpeptones which are hydrolysates of casein. A number of these nitrogen sources have been examined for cyclosporin production both in the presence and absence of inorganic nitrogen.

3.4.2.1 Determination of Optimum Nitrogen Source

An experiment was carried out to compare cyclosporin production on a complex nitrogen source, casamino acids (Agathos' medium), compared with that nitrogen source supplemented with 0.5% nitrate (Foster's medium). The results indicated that, for the top five producing strains, added nitrate did not increase cyclosporin production, rather cyclosporin production in these flasks was lower (Table 7). The addition of ammonium (NH_4OH) or any commonly used inorganic nitrogen source to the cultures did not increase cyclosporin production (Alan Jones, personal communication). Therefore the only nitrogen sources tested were organic caseinpeptones.

A number of nitrogen sources, all digests of casein, were examined for cyclosporin production with a sorbose carbon source. These included casamino acids, vitamin assay casamino acids, Bacto-Casitone, Bacto-Peptone, Tryptone, and a negative control. All four nitrogen sources showed very good fungal growth (9-14 g/L) except the casamino acids and the negative control (>2 g/L). Cyclosporin production for the vitamin assay casamino acids, Bacto-Casitone, Bacto-Peptone, and Tryptone were all statistically equivalent, yielding 110-156 mg/L mean cyclosporin (Table 11). The ethyl acetate extracts from each of the cultures had variable separation of the organic and aqueous phases. Vitamin assay casamino acids had the cleanest separation of the phases and the HPLC profile from vitamin assay casamino acids culture extracts had the fewest extraneous peaks of any of the nitrogen sources tested. For these reasons, as well as the high cyclosporin production,

Table 11 Cyclosporin Production on a Variety on Nitrogen Sources

Nitrogen Source	Final pH	Mycelial Dry Weight (g/L)	Cyclosporin Production(mg/L) (± S.D)
Negative Control	4.5	1.1	23 (2)
Casamino Acids	5.1	1.8	65 (16)
Vitamin Assay Casamino Acids	4.4	13.7	156 (6)
Bacto-Casitone	5.0	12.6	121 (40)
Bacto-Peptone	5.1	8.6	119 (31)
Tryptone	5.6	9.2	111 (18)

Precultures were started in 100 mL of SVA medium inoculated with 3.25 x 10^5 spores of *T. niveum* UAMH 2472 from glycerol stocks. These precultures were incubated 185 h, then were pooled and homogenized. Inoculations of 10 mL were made into triplicate flasks of each of the following sorbose-based medium (2% sorbose,1% nitrogen source, 1% KH_2PO_4, and 0.5% KCl); no nitrogen source, casamino acids, vitamin assay casamino acids, Bacto-Casitone, Bacto-Peptone, and Tryptone. Note the negative control and casamino acids were only carried out in duplicate. The production cultures were incubated for 15 days then homogenized and analyzed.

vitamin assay casamino acids were used as the nitrogen source for all future experiments unless otherwise specified.

The experiments to determine the optimum nitrogen source all examined these nitrogen sources at the 1% concentration. Whether this concentration was optimum for fungal growth and cyclosporin production was unknown. To resolve this question an experiment was carried out to determine cyclosporin production with five concentrations of vitamin assay casamino acids. These were tested, along with a negative control, with *T. niveum* UAMH 2472 in the sorbose medium. The results (Figure 10) demonstrated that final cyclosporin production was very low with low vitamin assay casamino acids concentration but increased as the nitrogen source increased. The optimum nitrogen source concentration appeared to be 10 g/L. This was confirmed by Duncan's Multiple Range analysis which showed the 1.0% and 5.0% vitamin assay casamino acids to be equivalent but superior to the other concentrations. For convenience as well as cost efficiency the nitrogen source was used at the 1% concentration.

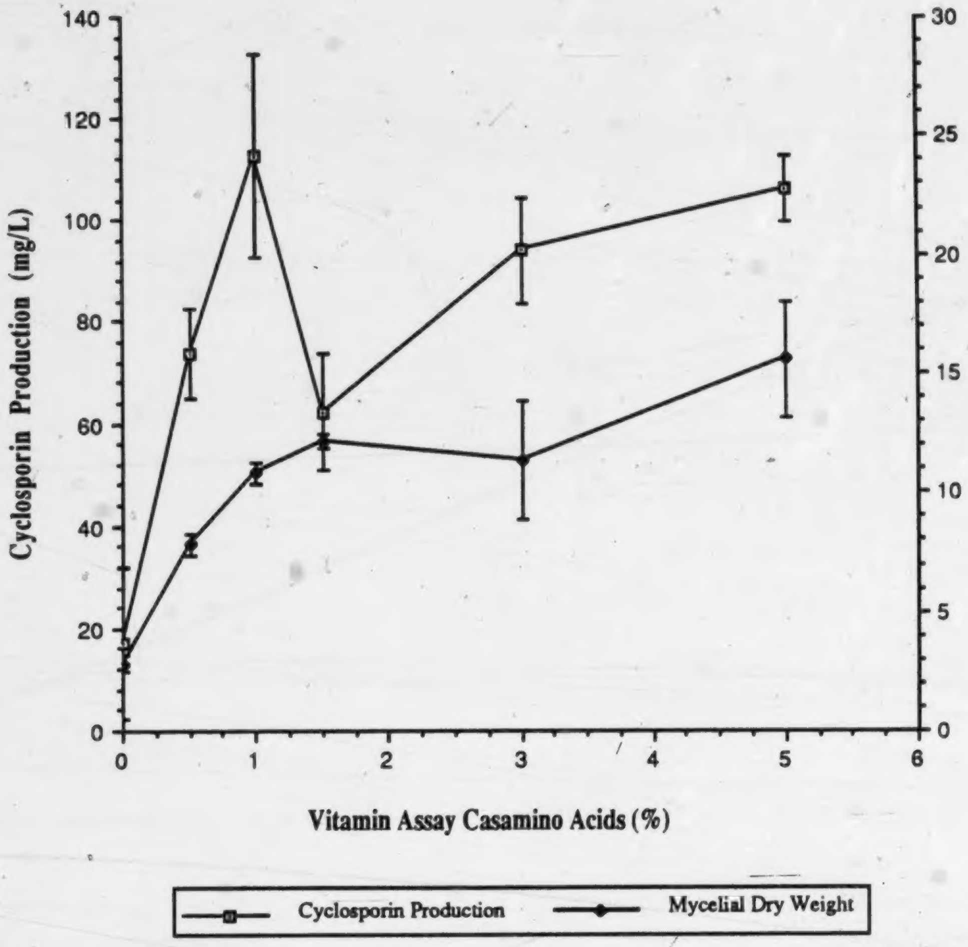
3.4.2.2 Vitamin Assay Casamino Acids Preparation

Cyclosporin production at this point of the research was still sufficiently inconsistent that multiple flasks were necessary so attempts were made to investigate likely variables. It had been observed that the method of sorbose sterilization affected the cyclosporin production of the medium. For this reason several aspects of nitrogen source preparation were investigated.

The first of these factors was the method of sterilization. It was observed that there was little difference in cyclosporin production between filter-sterilized sorbose and autoclaved sorbose. To ensure that there were no heat-labile amino acids in the vitamin assay casamino acids that would be degraded during autoclaving, a solution of concentrated vitamin assay casamino acids was filter-sterilized then added to autoclaved medium. The

Figure 10 Effect of Increasing Vitamin Assay Casamino Acids Concentration on Cyclosporin Production

Precultures were prepared by inoculating 100 mL of SVA medium with 10^8 spores of *T. niveum* UAMH 2472 and incubating 72 h. 10 mL of preculture was inoculated into each of quintuplicate flasks containing SVA medium with different concentrations of vitamin assay casamino acids. These production cultures were incubated 15 days, made up to original volume with distilled water, homogenized, and analyzed.


Cyclosporin Production (mg/L)
0
20
40
60
80
100
120
140
Mycelial Dry Weight (g/L)
0
5
10
15
20
25
30
0
1
2
3
4
5
6
Vitamin Assay Casamino Acids (%)
Cyclosporin Production
Mycelial Dry Weight

control for this experiment was *in situ* autoclaved vitamin assay casamino acids. Additionally the sorbose content of the cultures was periodically sampled to monitor the progress of the fermentation. The results (top of Table 12) indicate that *in situ* autoclaving vitamin assay casamino acids yielded statistically higher cyclosporin levels than filter-sterilizing. This difference was not as marked as that observed between sorbose treatments but it was evident. The differences between the flasks was reduced somewhat as the cyclosporin levels produced were lower than previously obtained. Obviously there is formation or modification of some product during autoclaving sorbose and vitamin assay casamino acids that stimulated cyclosporin production. The exact nature of this product has not been determined. The sorbose concentration detected by the chemical assay in the *in situ* flasks was again only one-half that of the filter-sterilized flasks. Sorbose was quickly taken up by the mycelia and the levels on day 15 were only 1% of those initially observed.

One other factor that was examined in this experiment was the possible variations between different lots of vitamin assay casamino acids. An expired lot (Oct. 86) was used in the experiment just described and was found to be statistically different from the Apr. 1991 lot. Variations in amino acid content of vitamin assay casamino acids had been shown to occur among lots (Nolan, 1971). This might have had an effect on cyclosporin production as the previous experiment demonstrated. An experiment was carried out comparing four lots of vitamin assay casamino acids for cyclosporin production, mycelial dry weight, and pH. The results indicated that there was a difference between lots for cyclosporin production though the mycelial mass did not vary greatly among these cultures (Table 12). Duncan's Multiple Range test showed the lots Oct. 1992 and Jun. 1992 to be statistically superior to the Oct. 1986 and Apr. 1991 lots. Lot analyses of these four were obtained from the supplier (Difco) and compared to determine if any single factor could be responsible for the possible variation among lots. These analyses showed no significant differences between them. As the April 1991 lot had previously yielded higher levels of

Table 12 Cyclosporin Production on Different Lots of Vitamin Assay Casamino Acids

Lot Date	Final pH	Mycelial Dry Weight (g/L) (± S.D.)	Cyclosporin Prod. (mg/L) (± S.D.)	Specific Cyclosporin Prod. (mg/g) (± S.D.)
Apr.1991*	5.6	8.0 (0.0)	73 (11)	9.1 (1.4)
Apr. 1991	5.4	8.9 (0.0)	88 (4)	9.8 (0.5)
Oct. 1986	5.8	6.0 (0.3)	71 (5)	12.0 (1.0)
Oct. 1986	5.3	8.9 (0.5)	78 (20)	8.8 (2.5)
Apr. 1991	4.5	9.9 (0.2)	78 (5)	7.9 (0.6)
Jun. 1992	4.4	10.3 (0.2)	96 (2)	9.3 (0.3)
Oct. 1992	5.4	9.4 (0.7)	116 (12)	12.5 (1.3)

*= filter sterilized through Millex GS (0.22μm) filter

These results are from two experiments. The first experiment (row 1-3) was started from precultures of 100 mL SVA medium inoculated with 10^8 spores from glycerol stocks of *T. niveum* UAMH 2472 and incubated 96 h. These precultures were pooled and 10 % inoculations were made into the following SVA-based medium: quintuplicate flasks of medium with filter-sterilized (through Millex GS filter, 0.22 μm) April 1991 vitamin assay casamino acids added, quintuplicate flasks of SVA medium made up with Oct. 1986 vitamin casamino acids, autoclaved *in situ* and triplicate flasks of SVA medium made up with April 1991 vitamin assay casamino acids. autoclaved *in situ* These cultures were incubated 15 days, made up to original volume with distilled water, homogenized, then analyzed.

The second experiment (rows 4-7) was carried out as described above except the production media was SVA medium using these nitrogen sources: Oct. 1986 (3 flasks), April 1991 (5 flasks), June 1992 (3 flasks), and Oct. 1992 (3 flasks) all autoclaved *in situ*.

cyclosporin production (130 mg/L) and the two high yielding lots had been freshly opened, there may be a decrease in cyclosporin production over the time the bottle of vitamin assay casamino acids had been open. This should be further investigated.

It can be concluded that the nature of the nitrogen source has a definite effect on cyclosporin production. Not only must the optimum nitrogen source be selected from the great number of those available, but the optimum concentration, method of preparation, and even lot number of that nitrogen source should be determined. All this was necessary in order to produce the highest possible cyclosporin levels. There are several nitrogen sources utilized in the literature which were unavailable for testing such as the "caseinpeptone" used by Sandoz workers. However while continued investigation may eventually determine a better nitrogen source than vitamin assay casamino acids, in terms of cyclosporin production, ease of product separation, and lack of coextracting metabolites, vitamin assay casamino acids have proved very satisfactory.

3.4.3 Optimization of Medium Salts for Cyclosporin Production

The salts component of the growth and production media might also be altered to yield higher levels of cyclosporin. One of the primary functions of the salts is to provide a high buffering capacity to regulate large pH fluctuations which might occur during fermentation. The addition of sufficient concentrations of phosphate usually accomplishes this task. Other salts, including those of cations which are normally required by cells and required by the fermentation to produce the final product are added. The effect of these added salts must be considered and in the case of final product metabolites, optimized, to produce the highest levels of that product.

Several media have been described in the literature for the production of cyclosporins. All use different combinations of salts as well as carbon source and nitrogen sources. To determine which of these media would be best for our particular strains, several were tested. This was carried out in the comparison of carbon sources in different media (Table 7) comparing Agathos' medium with Foster's medium. As stated earlier Duncan' Multiple Range analysis did not show Agathos' medium to be superior for cyclosporin production but considerations such as reproducibility between replicate samples pointed to the use of Agathos' medium with a sorbose carbon source.

Once the medium of choice had been established the components of that medium should be altered in order to find the best combination for optimum cyclosporin production. These components, in the case of Agathos' medium, were the KCl and KH_2PO_4. The KH_2PO_4 was required for nucleic acid synthesis and for its buffering capacity. The KCl had likely been added for its ability to stabilize κ-carrageenan. Whether these two components were absolutely necessary for cyclosporin production was investigated.

The first of these two to be considered was KCl. This salt first appeared in cyclosporin production medium in the 1983 paper by Foster *et al.* where it was added to stabilize the κ-carrageenan immobilized *T. inflatum* pellets. Also the increased ionic strength helped to reduce foaming of the fermentation in an airlift fermenter. As the majority of this study was not carried out using immobilized *Tolypocladium sp.*, and subsequent immobilizations using κ-carrageenan were unsuccessful, experiments were carried out to determine if added KCl was necessary for cyclosporin fermentations. One experiment compared flasks of sorbose medium with and without added KCl for cyclosporin production. The results indicated that the two treatments were not statistically different. The means were 124 ± 24 mg/L with added KCl and 125 ± 11 mg/L without added KCl. All other parameters such mycelial dry weight, pH and specific production were virtually identical. As vitamin assay casamino acids contain 38% NaCl the addition of KCl is unlikely to have as profound effect on these cultures as casamino acids (14% NaCl) medium.

A further experiment compared cyclosporin production using sorbose medium with no added KCl or $CaCl_2$, added KCl, and added $CaCl_2$. Addition of $CaCl_2$ was investigated as Ca^{++} is necessary for stability of alginate. Alginate immobilization was suggested for *T. niveum* due to the relative failure of carrageenan immobilization. The flasks with added $CaCl_2$ formed a chalky white precipitate after autoclaving. This precipitate was likely $(Ca)_3(PO_4)_2$ and disappeared by day 15, likely due to acid production during the fermentation. Cyclosporin production in the flasks with and without added KCl were again not statistically different, being 135 ± 24 mg/L with added KCl and 138 ±18 mg/L with no added KCl. The flasks with added $CaCl_2$ had mean cyclosporin production of only 64 ±10 mg/L. All of these flasks demonstrated good mycelial growth, 8.2-10.3 g/L dry weight. The results from these experiments demonstrate that the addition of KCl had little effect on cyclosporin production but that the addition of $CaCl_2$ seriously reduced cyclosporin production. For this reason alone alginate immobilization was not advisable for *T. niveum* as addition of the stabilizing cation reduced cyclosporin production by one-half.

The other salt added to the growth medium was KH_2PO_4. Experiments on phosphate concentrations in other systems led to the observation that high phosphate levels could inhibit secondary metabolite production (Creuger and Creuger, 1984). Also a great number of different phosphate levels have been reported in the literature with varying cyclosporin production. An experiment was carried out examining cyclosporin production at a number of phosphate concentrations. The results shown in Table 13 indicate that cyclosporin production for all cultures was quite high as was the mycelial growth. Duncan's Multiple Range test showed that the lower phosphate concentrations (0.1, 0.5, and 1.0 mM) were different and superior to the higher concentrations. Because a control was not run, the experiment was repeated.

All the cyclosporin levels were low in the repeated experiment including the control

Table 13 Effect of Phosphate Concentration on Cyclosporin Production

Added Phosphate (mM)	Final pH	Mycelial Dry Weight (g/L) (± S.D.)	Cyclosporin Prod. (mg/L) (± S.D.)	Specific Cyclosporin Prod. (mg/g) (± S.D.)
0.1	5.9	8.1 (0.2)	109 (1)	13.5 (0.3)
0.5	6.0	8.4 (0)	116 (11)	14 (1)
1.0	5.9	8.3 (0.3)	109 (4)	13.2 (1)
5.0	5.8	8.2 (0)	103 (0)	12.6 (0)
10.0	5.7	8.1 (0.2)	102 (7)	12.6 (1)
15.8	5.5	8.4 (0)	96 (7)	11.3 (0.9)
0	6.1	7.5 (0.1)	56 (4)	7.4 (0.1)
1.0	6.2	7.5 (0.3)	66 (2)	8.9 (0.5)
5.0	6.0	7.6 (0.1)	50 (1)	6.6 (0.1)
10.0	5.9	7.8 (0.2)	48 (11)	6.1 (1.2)
74.0 (control)	5.3	8.9 (0.5)	78 (20)	8.8 (2.5)

These results are from two experiments using the same experiemental protocol but with different phosphate concentrations. The protocol involved inoculating preculture flasks with 10^8 spore of *T. niveum* UAMH 2472 into 100 mL of SVA medium (1% KH_2PO_4 (74 mM)) and incubating 96 h. 10 mL of these precultures were inoculated into 100 mL of SVA medium with the appropriate phosphate concentration. All cultures were carried out in triplicate. The cultures were incubated 15 days, maded up to original volume with distilled water, homogenized, and analyzed.

(78 ± 20 mg/L) which had the highest cyclosporin production (Table 13). The low cyclosporin levels indicate that further work should be carried out in this direction. An interesting observation was that as the phosphate levels decreased, the final pH of the fermentation increased. The reason for this was likely due to the decreased buffering capacity of the medium with lowered phosphate concentration. An experiment with varied phosphate concentrations and pH was carried out to further elucidate this. These results (Table 14) indicate that there was no phosphate inhibition even at very high concentrations (300 mM). However phosphate concentrations of 74 mM (control) and 200 mM yielded statistically superior production and as such 74 mM was used for all remaining experiments. Initial pH also had little effect on the production of cyclosporin over the range of pH 4.2-6.2. However pH of 4.2 and 5.2 (control) yielded statistically higher cyclosporin levels. The optimum condition for cyclosporin production was 74 mM KH_2PO_4 at pH 5.2.

The conclusion from these experiments on optimization of the salts component of the medium for cyclosporin production showed that added KCl was not necessary for high cyclosporin production but it may prove beneficial in reducing foaming during fermentation by increasing the ionic strength of the medium. KCl continued to be added to the medium though these ions were already present in KH_2PO_4 and vitamin assay casamino acids (38% NaCl).

3.5 Procedural Considerations

A large proportion of the work carried out in this project has been directed toward obtaining reproducibly high levels of cyclosporin. The emphasis has been on reproducibility of these levels and throughout attempts have been made to standardize the procedure wherever possible. A number of these standardized procedures were not instituted until later in the project. For this reason the methods used in some early

Table 14 Effect of Initial pH and Phosphate Concentration on Cyclosporin Production

Initial pH	Phosphate Conc. (mM)	Final pH	Mycelial Dry Weight (g/L) (± S.D.)	Cyclosporin Prod. (mg/L) (± S.D.)	Specific Cyclosporin Prod. (mg/g)
4.2	74	3.7	8.9 (0.3)	123 (7)	13.9
4.7	74	4.1	10.4 (0.2)	105 (4)	10.1
5.2	74	4.5	9.6 (0.1)	129 (3)	13.4
5.7	74	4.9	8.8 (0.1)	111 (11)	12.6
6.2	74	5.6	7.6 (0.1)	85 (9)	11.1
5.2	300	4.9	9.5 (0.4)	122 (4)	12.9
5.2	200	4.8	9.5 (0.1)	101 (7)	10.7
5.2	74	4.5	9.6 (0.1)	129 (3)	13.4
5.2	50	4.3	9.6 (0.3)	103 (3)	10.7
5.2	10	4.2	9.9 (0.5)	84 (7)	8.4

Precultures were started by inoculating 10^8 spores of *T. niveum* UAMH 2472 into 100 mL of SVA medium and incubating 96 h. The precultures were homogenized, then 10 mL of the pooled preculture were inoculated into triplicate production flasks containing SVA medium with varied initial pH and phosphate concentrations. These cultures were incubated 10 days, made up to original volume with distilled water, homogenized, and analyzed.

experiments differed from those used in later ones, and may explain some of the differences between results obtained for similar experiments.

One of the first modifications in the methods was homogenization of the production cultures after incubation. Initial experiments demonstrated that the cultures were composed primarily of clumps and pellets of mycelia which did not pipette evenly nor extract reproducibly. For these reasons the cultures were homogenized to ensure the cultures were homogeneous for analysis. The precultures were also homogenized (if not already homogeneous) to ensure that each of the production flasks received a similar inoculum.

Homogenization of the production cultures was only one of the steps taken to allow comparison of different experiments. Another step became obvious after it was observed that the final volumes of the production flasks were not always the same for different flasks, especially those with different mycelial growth or morphology. To alleviate this problem, the contents of all flasks were made up to their original volume with distilled water prior to homogenization which eliminated any variation due to unequal evaporation. However this dilution of the cultures served to decrease the final volumetric production which of course diminished the values obtained relative to those in the literature.

Another area of difficulty which was encountered during the project was the extraction of cyclosporins from the culture. The usual precautions such as extraction of internal standards alone or added to producing cultures revealed essentially 100% recovery of the added cyclosporins (cyclosporin D was the internal standard used). However it was noted that the final volume of the organic solvent was slightly less (5-10%) than the initial volume. It was thought that this loss was due to evaporation of the ethyl acetate during the overnight extraction. For a period of time the volume of the organic phase was made up to original volume with added ethyl acetate. However it was considered that volume might not be the most accurate parameter to judge loss of the organic phase. The mass of the extractions was then monitored. An experiment was carried out on a homogeneous culture (derived from one of the 2L airlift fermenter runs discussed) whereby ten replicate extractions were carried out. These extractions were weighed before and after the overnight agitation and then analyzed for cyclosporin content. The results indicated that the ten extractions lost an average of 0.06 ± 0.03 g. This represents a loss of less than 0.7% of the added ethyl acetate's average mass of 8.99 g. Obviously there was no significant loss from the extraction bottles. The loss in volume was likely a result of partition of ethyl acetate into the aqueous phase and interaction with the biomass. Therefore the organic phase did not have to be made up to volume and could be used straight from the extraction bottle. Analysis of these extractions for cyclosporin content by HPLC revealed that none of the ten samples was statistically different from the others.

These experiments and the standardization of the procedure served to increase the confidence in the data obtained. The preceding experiment, where ten samples were shown to have no difference, indicated that any difference found between cultures or treatments were due solely to the fungal activity. There was no appreciable error in the methods, so differences among the cultures must be a result of the cultures themselves, not the analytical procedures.

The cultures will obviously have differences among them. Whether or not these differences were significant had to be determined. Several factors in the methodology such as preculture inocula and homogenization of precultures had been standardized. To determine if replicate flasks could be safely considered as a group, that is with only the expected deviation between them, an experiment was carried out with five flasks of 2% sorbose medium inoculated from a common preculture. These five flasks were found to have an average cyclosporin production of 207 ± 15 mg/L. The standard deviation, 15 mg/L was less than 7% of the mean and the range of values was only 34 mg/L. These results indicated that the reproducibility of cyclosporin production within a group of replicate flasks was acceptable.

These experiments and modifications to the methodology have hopefully served to make the results valid and consistent throughout. The validity of values obtained from fungal cultures must always be proved to be reproducible as there is such a large degree of variability within fungal fermentations. The modifications appear to have shown that any data obtained were valid and that differences within an experiment were due to the fungal activities and not a result of the methods used.

3.6 Medium Supplementation

As discussed in the literature review, Kobel and Traber (1982) supplemented their growth medium with an excess of constituent amino acids in order to direct the synthesis of cyclosporins to a particular class. They were successful in this and as an unexpected benefit were able to produce higher cyclosporin levels than had previously been observed. This directed synthesis was carried out using a mutant of the parent strain of *T. inflatum* NRRL 8044. An experiment was carried out to determine if this directed synthesis could be carried out using the highest producing strains of *T. niveum* found in the screening experiments. Additionally it had been found from both the literature and from experimentation that methylation of the molecule that occurred after cyclization (Kobel *et al.* 1983). In order to enhance cyclosporin production, a number of methyl group donors were added to the medium to facilitate this methylation.

3.6.1 Directed Synthesis

The directed synthesis of cyclosporins was carried out for a mutant derived from *T. inflatum* NRRL 8044 by Kobel and Taber (1983). An similar experiment was carried out using the five highest cyclosporin producing strains of *T. niveum* and a medium based on that of Agathos' *et al.* (1986) which was supplemented with 8 g/L of one of four substituent amino acids. The results of this experiment are shown in Table 15. From this

Table 15 Directed Synthesis of Cyclosporin Production in Five *T. niveum* Strains

Precursor (8 g/L)	Mycelial Dry Weight (g/L)	Total Cyclosporin (mg/L)	Single compound(% of total cyclosporin)			
			A	B	C	G[a]
T. niveum 2472						
Control	7.0	91	78	5	17	0
DL-α-Abu	7.0	48	100	0	0	0
L-Ala	6.7	68	79	9	12	0
L-Val	7.3	119	92	3	5	0
DL-Nva	6.7	66	24	0	0	76
T. niveum 2880						
Control	10.0	112	90	2	8	0
DL-α-Abu	6.3	37	100	0	0	0
L-Ala	6.3	69	76	10	14	0
L-Val	7.0	120	90	5	5	0
DL-Nva	5.7	81	23	0	0	77
T. niveum 4002						
Control	6.3	130	75	12	13	0
DL-α-Abu	4.3	71	100	0	0	0
L-Ala	5.3	53	78	12	10	0
L-Val	4.3	120	90	5	5	0
DL-Nva	6.3	64	31	3	2	65
T. niveum 4594						
Control	8.7	232	84	4	12	0
DL-α-Abu	7.3	50	100	0	0	0
L-Ala	9.7	209	72	14	14	0
L-Val	7.3	154	100	0	0	0
DL-Nva	8.7	212	36	0	7	57
T. niveum 4828						
Control	8.0	148	75	10	15	0
DL-α-Abu	5.7	23	100	0	0	0
L-Ala	8.0	104	70	18	12	0
L-Val	6.3	155	97	1	2	0
DL-Nva	7.3	57	35	0	8	57

[a]=tentatively identified

Precultures were started from spores suspensions washed from sporulating plates of each strain. These spore suspensions were inoculated into 125 mL flasks containing 25 mL of medium (2% sorbose, 1% casamino acids, 0.5% KH_2PO_4, and 0.25% KCl). These precultures were incubated 7 days then homogenized. The homogenized preculture was inoculated into duplicate flasks containing 100 mL of medium with the appropriate added amino acid. These production flasks were incubated 15 days then homogenized. After homogenization the cultures were analyzed.

table it can be seen that cyclosporin production had indeed been directed to several classes. The addition of DL-α-aminobutyric acid, the position 2 amino acid of cyclosporin A directed synthesis 100% to cyclosporin A in all five strains, however the total cyclosporin produced was reduced. The addition of L-alanine resulted in an increase in the amount of cyclosporin B produced (which has L-alanine at position 2). This increase was most dramatic in *T. niveum* UAMH 2880 and 4594 where a 3-fold increase in cyclosporin B was observed. Addition of L-valine did not result in the expected increase in cyclosporin D formation (L-valine is the position 2 amino acid in cyclosporin D) but rather an increase in cyclosporin A produced. In the case of *T. niveum* UAMH 4594 the addition of L-valine directed synthesis 100% to cyclosporin A. The reasons for synthesis being directed to cyclosporin A rather than D were unclear but may be related to the general non-specificity of peptide antibiotic synthesis and the closely related structures of L-valine and L-α-aminobutyric acid which differ by a single methyl group which might be cleaved off during the fermentation. The cyclosporins synthesized when DL-norvaline was added was much simpler. L-norvaline is the substituent amino acid at position 2 of cyclosporin G and as expected cyclosporin G was produced by all strains when this amino acid was added. It should be noted that this particular class of cyclosporin had not been previously observed and direction of synthesis to this class ranged between 57-77% of cyclosporins produced. In the case of *T. niveum* UAMH 4594 total production of cyclosporins was 212 mg/L of which 57% was cyclosporin G with remainder cyclosporin A. (Note that identification of cyclosporin G was based on the order of elution of peaks on HPLC analysis. To rigorously determine the identity of this cyclosporin peak further analysis would be required.)

This experiment clearly showed that cyclosporin production could be directed for these strains of *T. niveum*. Cyclosporin production in all the control flasks was reasonably high (91mg/L) to very high for strain 4594 (232 mg/L). The addition of some amino acids, particularly DL-α-aminobutyric acid reduced total cyclosporin production while others such as L-valine both increased total production and directed synthesis to the commercially useful class, cyclosporin A. It was noted that for each strain there were differing responses to the amino acids concerning total production, but the trend of directed synthesis was constant throughout all five strains.

3.6.2 Supplementation with Methyl Donors

The rapid uptake of ^{14}C-methyl-methionine (see section 3.8) and the post-cyclization methylation posed the concern of methylation as being the limiting step in cyclosporin production. For these reasons, supplementation of the growth medium with methyl donating compounds was suggested to increase cyclosporin production. A series of previously untested methylating compounds were added in high concentration (8 g/L) to the production cultures and the cyclosporin production of these cultures was monitored. The results demonstrated that addition of these compounds did not dramatically altered cyclosporin production or other growth parameters. The addition of carnitine or choline to the cultures demonstrated the highest cyclosporin production for any of the methylators (70-80 mg/L);(betaine, N,N,-Dimethyl glycine, carnitine, and choline) but the negative control yielded the highest levels observed (120 mg/L). It appears then that the addition of some methyl-donating compounds did not increase cyclosporin production.

3.7 Time Course Experiments

The monitoring of cyclosporin production as well as the growth of the fungus is of great interest in optimizing cyclosporin production. Determining the appropriate harvest time is best found by a time course study. However some problems present themselves when monitoring the growth of fungal cultures. The most obvious indicators of growth in a bacterial cultures such as turbidity and light-scattering are not easily determined in fungal cultures due to the heterogeneous nature of a growing organism. Some portions of the hyphae are very metabolically active while others are inactive. The most convenient measure of growth, dry cell weight is not necessarily indicative of growth as the hyphal walls may continue to thicken, increasing cell weight, while there is no real growth occurring. For this reason RNA and protein determinations are better indications of growth but are difficult to carry out as the hyphae must be broken open to gain access to the cellular components. In the case of *Tolypocladium*, breaking the hyphal walls proved too difficult, therefore mycelial dry weight was used as a relative indicator of cell growth, imprecise as it may be. Other fermentation parameters were quantitated in some of the time course experiments carried out.

3.7.1 Time Course Experiments at 27°C

The initial screening of ten *T. niveum* strains involved a study of cyclosporin production over time. The results of this experiment were used primarily to determine the highest producing strains of *T. niveum* but the course of cyclosporin production for each strain also was found. Due to the methods of sampling, analysis, and quantitation used in this experiment which was carried out at the start of the project, the results must be taken with some reservations. The general trend observed was that cyclosporin was present by day 7 when sampling started and continued to increase until day 15 when sampling was halted. For some of the strains, cyclosporin levels began to decline after day 14. From this initial experiment *T. niveum* cultures were harvested on day 15 in all future experiments.

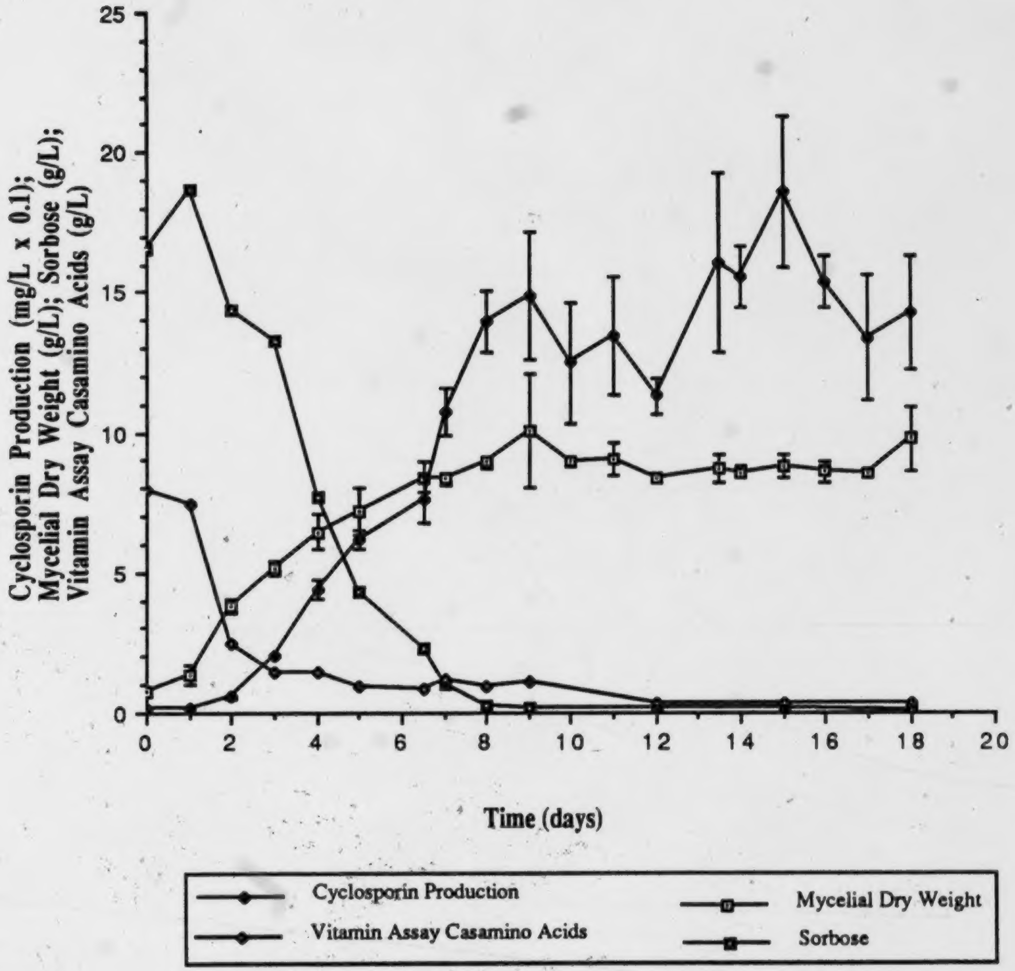
The first organized time-course experiment was carried out with *T. niveum* cultures started from spore suspensions with no preculture step. One of the five high producing strains of *T. niveum*, UAMH 4594, was selected and each of 30 flasks containing 1.6% sorbose medium (1% casamino acids) was inoculated with 6.5 x 10^5 spores from glycerol spore stocks of the fungus. The size of this inoculum has since been shown to be too small. However at the time of the experiment the optimum spore inoculum was unknown. The low spore inoculum resulted in a slow cyclosporin increase at a constant rate until approximately day 17 when production ceased at 40-50 mg/L. The rate of increase in cyclosporin levels (3.1 mg/L/day initially) was quite slow compared to the initial time course experiment where cyclosporin production had peaked by day 14. The most obvious reason for this is that the initial experiment was carried with cultures started from preculture and the spore inoculum for these precultures was higher than the 10^5 spores inoculated into these cultures. The effect of the small inoculum was also apparent in the growth of the fungus. The final mycelial dry weight was only 2-3 g/L which was significantly lower than the previously observed 7-10 g/L. The low growth of the fungus might have also been due in part to the fact casamino acids was used as the nitrogen source rather than the superior vitamin assay casamino acids. The conclusion from this experiment was that cyclosporin levels increase with the increase in mycelial dry weight and that cyclosporin is produced from day 3 unlike traditional secondary metabolites. Other parameters of the fermentation must also be examined such as sorbose utilization and uptake of casamino acids.

The next time course study was carried out more closely followed the methods used for a typical cyclosporin fermentation. Again a small spore inoculum was used, 3.25 x 10^5 spores from glycerol spore stocks of *T. niveum* UAMH 2472 but this was inoculated into a preculture which was incubated 7 days then 10 mL of this preculture was inoculated into each of 16 flasks containing 2% sorbose medium-1% casamino acids. The cyclosporin production increased slowly for the first 7 days then increased rapidly to a peak of 78 mg/L . The mycelial dry weight was low throughout, never increasing higher than 2 g/L. This poor growth had been noted in the previous time course experiment as had the lowered cyclosporin levels. In this case, the reason was likely the use of casamino acids as the nitrogen source which reduced mycelial growth both in the preculture and the production flasks. Additionally, the the initial spore inoculum was lower than what was subsequently found to be the optimum. For these reasons the cultures did not grow well or produce high levels of cyclosporin.

A final time-course study was carried out under current optimum conditions. Both the sorbose and vitamin assay casamino acids levels were monitored to give a better indication of medium changes during the fermentation. The precultures were inoculated with 10^8 spores of *T. niveum* UAMH 2472 from glycerol spore stocks and incubated 96 h. These precultures were used to inoculate 10 mL of preculture into 54 flasks of SVA medium. It should be noted that the precultures displayed very good growth (mycelial dry weight= 2.9 g/L) and cyclosporin production (45 mg/L) in the 96 h of incubation from inoculation. These triplicate production cultures were incubated, harvested, and analyzed. The results (Figure 11) indicated an initial 2 day lag in cyclosporin production followed by a rapid increase (20.4 mg/L/day) and finally a leveling of cyclosporin production at 150 mg/L. The improvement shown in this time course over previous attempts was the increase in rate of cyclosporin production and a final cyclosporin level that was twice as high as the previous experiment (150 mg/L versus 78 mg/L respectively). The mycelial dry weight increased (1.4 g/L/day) concurrently with cyclosporin production though mycelial growth leveled off (at a maximum of 8.5 g/L) slightly earlier than cyclosporin production did. The vitamin assay casamino acids levels decreased very rapidly, by day 3 there was only 1.5 g/L remaining in the supernatant from the original 10 g/L in the medium. This level remained constant until day 12 when virtually no vitamin assay casamino acids were detected in the supernatant. The uptake of sorbose was less rapid but just as complete. By day 5, 75% of the sorbose had been taken up from the supernatant. This was complete by day 8. For all of the processes described it appears a lag of one day was required for the fungus to acclimate and resume its metabolic activities prior to the onset of growth.

Figure 11 Production of Cyclosporin and Nutrient Uptake over Time in Shake Flask Culture by *T. niveum*

Five flasks containing 100 mL of SVA medium were each inoculated with 10^8 spores of *T. niveum* UAMH 2472 from glycerol spore stocks. These flasks were incubated 96 h, then pooled and diluted to 560 mL total volume. Fifty-four flasks, each containing 100 mL of SVA medium, were inoculated with 10 mL of preculture. At different times, triplicate flasks were harvested. Upon harvesting each culture was made up to original volume with distilled water, homogenized, and analyzed.


Cyclosporin Production (mg/L x 0.1); Mycelial Dry Weight (g/L); Sorbose (g/L); Vitamin Assay Casamino Acids (g/L)
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18
20
Time (days)
Cyclosporin Production
Vitamin Assay Casamino Acids
Mycelial Dry Weight
Sorbose

Attempts to determine RNA and protein content were inconsistent due to the difficulty in breaking down the hyphal walls and releasing the intercellular components. Several treatments involving boiling with NaOH, enzyme treatment (incubation with Driselase, Kyowa Hakko Kogya Co., Tokyo Japan), and solubilization were attempted but none served to consistently release the cytoplasmic components. From the results the optimum time for harvest of the production cultures remains 15 days as indicated by Figure 11. The pH of all these time course experiments remained quite constant through the course of the fermentation, holding between pH 5.0 and pH 5.5.

This final time-course experiment with *T. niveum* UAMH 2472 has shown that under the optimized conditions high levels of cyclosporin can be produced and the course of the fermentation monitored by parameters other than analyzing for cyclosporin content. Another parameter, such as mycelial dry weight, could serve as an indicator of cyclosporin content for as dry weight increased so did cyclosporin content. The specific production (Figure 12) remained constant after day 7 of the fermentation at 15-18 mg/g. One might expect that a similar time-course would be expected for the other four high cyclosporin-producing strains of *T. niveum*.

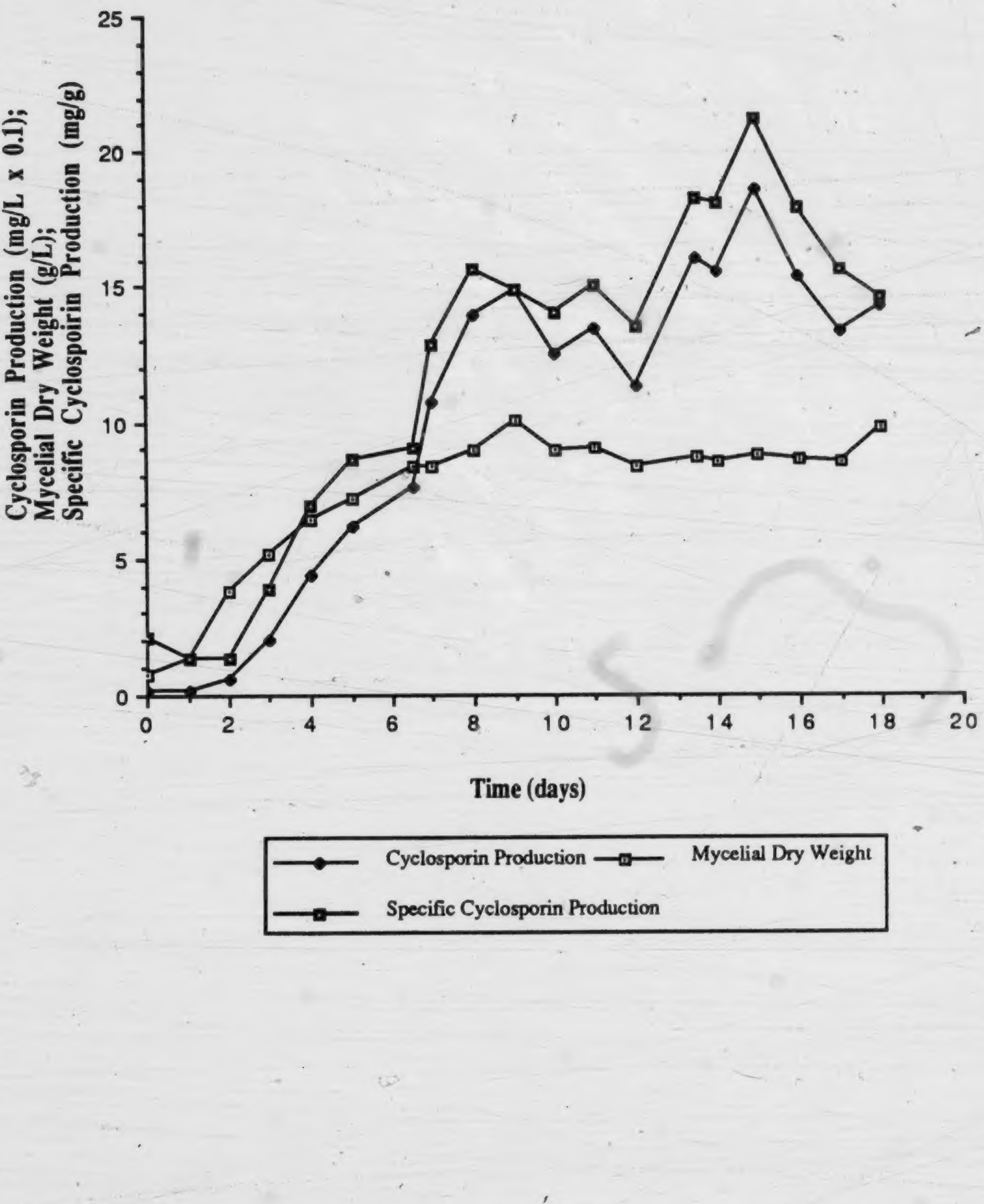
3.7.2 Time Course Experiments at 15°C

Agathos *et al.* (1986) indicated that cyclosporin production in *T. inflatum* (ATCC 34921) was 30% higher over 15 days at 15°C than at 27°C which is conventionally used for fungal cultures. This finding prompted questions pertaining to the effect of temperature on cyclosporin production as secondary metabolite production appears related to growth rate.

An experiment was carried out using three strains of *T. niveum* : UAMH 2472, 2880, and 4594. The precultures were prepared from spores washed from sporulating plates in 100 mL of 1.6% sorbose medium-1% casamino acids. After 7 days of incubation these precultures were used as a 1% inoculum for the flasks containing the 1.6% sorbose

Figure 12 Comparison of Mycelial Dry Weight, Cyclosporin Production, and Specific Cyclosporin Production over Time in Shake Flask Culture of *T. niveum*

The data was obtained from the cultures described in Figure 11.


Cyclosporin Production (mg/L x 0.1);
Mycelial Dry Weight (g/L);
Specific Cyclospoirin Production (mg/g)
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18
20
Time (days)
Cyclosporin Production
Mycelial Dry Weight
Specific Cyclosporin Production

medium. These flasks were harvested at appropriate times and analyzed. The results (Figure 13) showed that the cyclosporin production increased over time, but the time period was very long. Mycelial dry weight remained less than 2.5 g/L for all strains. The highest cyclosporin levels were seen at 588 h (24.5 days) which was much longer than observed in any other time course study. These levels of cyclosporin were indeed high, 156 mg/L for UAMH 2472 and 168 mg/L for UAMH 2880 but these levels were no higher than observed for 15 day cultures grown at 27°C. The low mycelial growth observed for all three strains had not been observed in high producing cultures. The very high specific cyclosporin production (75 mg/g) might be due to the reduced temperature and in part to the length of the fermentation.

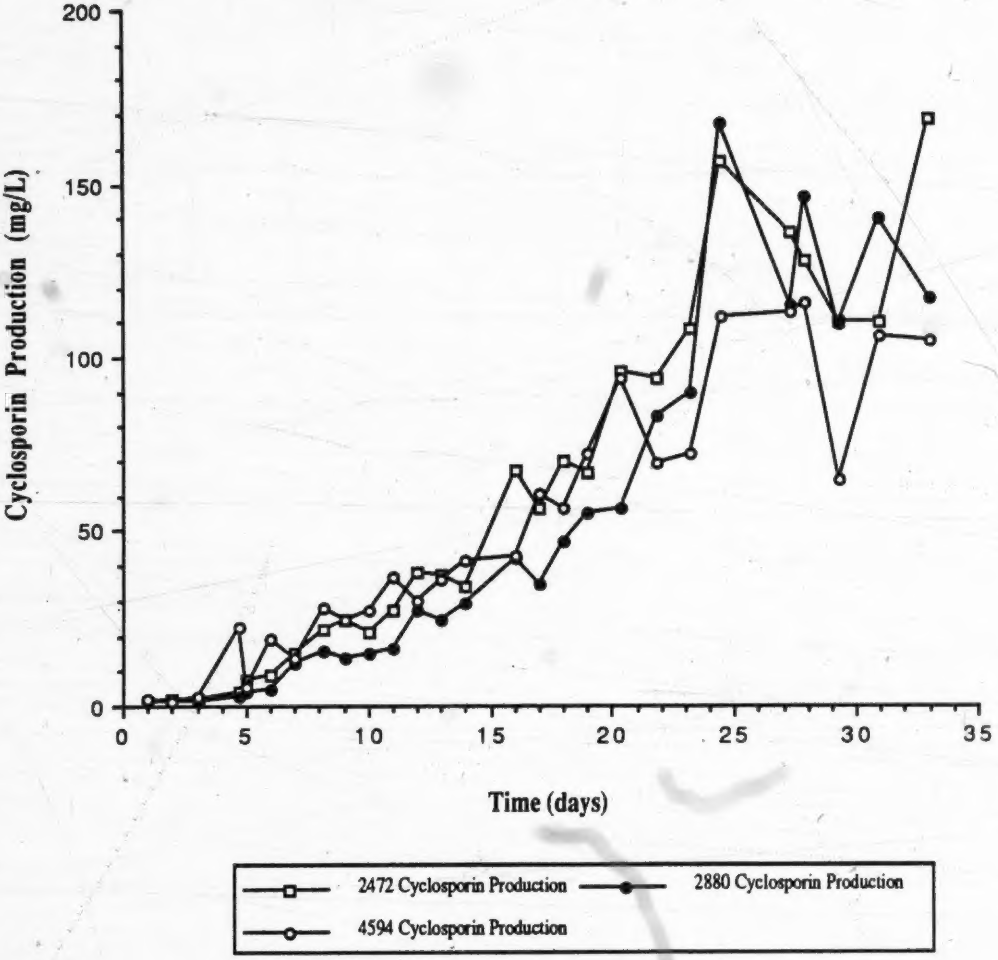
The results of this experiment indicated that cyclosporin production was not greatly improved by a reduction in temperature as cyclosporin levels at 17 days (384 h) were only 42 mg/L for strains 2880 and 67 mg/L for strain 2472 and 4594. Clearly these were less than the 130-150 mg/L ordinarily obtained after 15 days at 27°C. The high cyclosporin levels observed were likely due to the length of fermentation and a decrease in metabolic activity which might degrade any existing product. Thus growth at 15°C did not prove to be advantageous for cyclosporin production over growth at 27°C and in fact less cyclosporin was produced over 15 days for the UAMH strains of *Tolypocladium*.

3.8 Production of ^{14}C Labelled Cyclosporin

Production of radioactively labelled cyclosporins had been described by Kobel *et al* .(1983) and Zocher *et al*. (1984) using ^{14}C- and ^{13}C-labelled precursors. The best precursor for labelling had been found to be ^{14}C-methyl-methionine. Radiolabelled cyclosporin was prepared for two reasons. The first was to obtain information on the time-course of active synthesis and to determine if different feeding regimens produced different patterns of ^{14}C-cyclosporins. The second reason was to produce a ^{14}C-labelled

Figure 13 Effect of Incubation Temperature on Cyclosporin Production of Three Strains of *T. niveum* in Shake Flask Culture

Precultures for the 15° C flasks were prepared by washing sporulating plates of the respective *T. niveum* plates with distilled water and a 2% inoculum of this suspension was made into 100 mL of 1.6% sorbose-1% casamino acids medium. These precultures were incubated at 27°C for 7 days. One mL of preculture was inoculated into each of 30 flasks. These cultures were harvested at different times (one culture of each strain at each time point), homogenized, and analyzed.


200
150
100
50
0
Cyclosporin Production (mg/L)
0
5
10
15
20
25
30
35
Time (days)
2472 Cyclosporin Production
2880 Cyclosporin Production
4594 Cyclosporin Production

polypeptide (M.W.~1200 daltons) for use as a marker compound for gel exclusion chromatography by Dr. P.M. Fedorak.

Two feeding regimes were devised to produce ^{14}C-labelled cyclosporins. The results of these two methods can be seen in Figure 14 which describes the rapid uptake of the labelled methionine. Within 24-36 h, the radioactivity had become associated with the mycelium and only low counts could be detected in supernatant samples. Even the flasks to which 2.0 μCi had been added (method A) had only 10% of original radioactivity in the supernatant after 50 h. Radioactivity in the flasks which received 3 additions of the radiolabelled precursor (method B) was lost even more quickly, presumably as the smaller quantity of precursor was more quickly taken up by the larger quantities of biomass. HPLC analysis and scintillation counting of the final products indicated that the products from both methods had an retention time of 5-7 min, identical to authentic cyclosporin standards. HPLC analysis of ^{14}C-methyl-methionine indicated all activity was eluted in first two minutes, indicating that there was no ^{14}C-methyl-methionine in the final products.

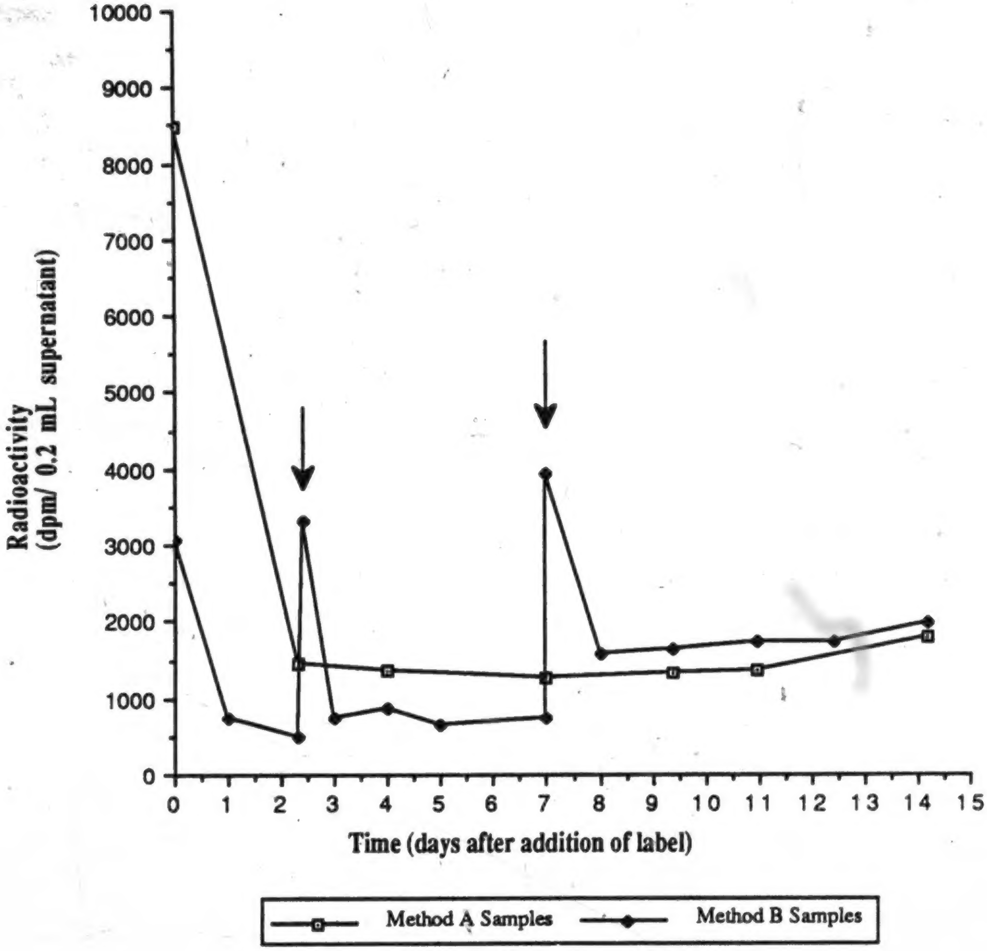
An accounting of radioactivity showed that a single addition of the precursor resulted in 29% incorporation of added precursor into the final product while the periodic addition method resulted in 20% incorporation. In both methods over 95% of the added activity was accounted for in either the product, mycelium, or growth medium. Method A yielded 83 mg cyclosporins (specific activity 7.8 x 10^4 dpm/mg) while method B yielded 84 mg cyclosporins (specific activity 5.3 x 10^4 dpm/mg). These products were mixtures of 60% cyclosporin A, 30% cyclosporin C, and 10% cyclosporin B.

3.9 Fermenter Production of Cyclosporin

Shake flask cultures are very useful for nutritional and physiological studies of a metabolite process. However to fully utilize the potential of an organism to produce a useful product, the process must be scaled up from shake flask cultures to produce

Figure 14 Uptake of ^{14}C-methyl-methionine by *T. niveum* UAMH 2472

Precultures were prepared by inoculating 10^8 spores of *T. niveum* UAMH 2472 from glycerol stocks to each of 5 flasks containing 100 mL of 2% sorbose-1% casamino acids medium. These precultures were incubated 7 days then pooled. Ten mL of pooled preculture was inoculated into each of five flasks containing 90 mL of SVA medium. After 72 h of incubation, half the flasks had 2.0 µCi of ^{14}C-methyl methionine added (Method A) and the other five had three additions of 0.66 µCi added at 72 h. (t=0 on graph) and where indicated by the arrows (Method B). Samples were withdrawn from each flask, centifuged and three samples of 0.2 mL of the supernatant were added to 10 mL of ACS™ scintillant and counted for ^{14}C activity. The average value from the three counts for each sample is shown.


10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Radioactivity
(dpm/ 0.2 mL supernatant)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Time (days after addition of label)
Method A Samples
Method B Samples

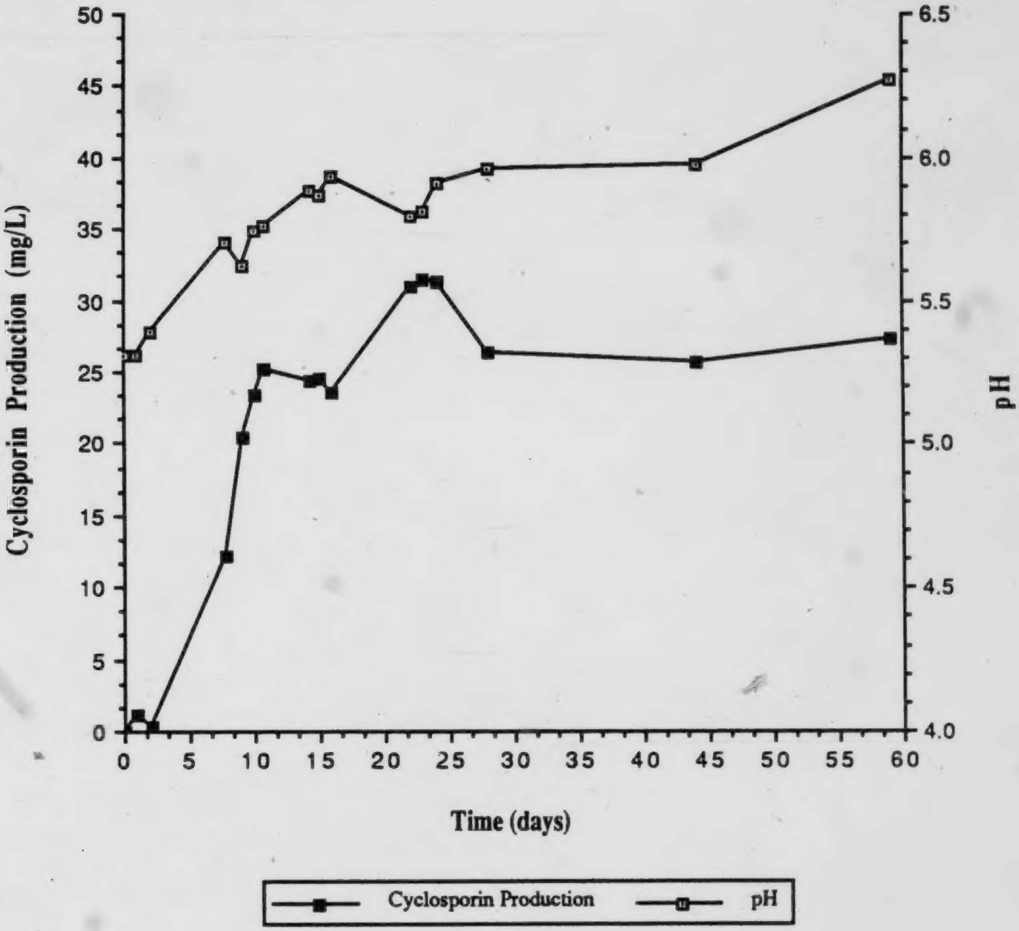
sufficient quantities of the product. Scale-up usually involves culturing the organism in increasingly larger fermenters. These fermenters may be of the stirred tank design which is the most commonly used or one of the airlift fermenter designs which are more readily scaled up. Scale-up is usually carried out in progressive steps and these experiments represent only the initial stages.

3.9.1 Stirred Tank Fermenter (10 L)

The stirred tank fermenter is the most commonly used design for industrial fermentations. Stirred tank fermenters have been successfully used for cyclosporin fermentations at the industrial level (Dreyfuss *et al.* 1976). A single experiment was carried out in a 10L Microferm™ stirred tank fermenter using *T. niveum* UAMH 4594 and a sub-optimum medium (1.6% sorbose-1%casamino acids). The results from this fermentation showed promise for scale up utilizing stirred tank fermenters. The results presented in Figure 15 show increasing cyclosporin levels for 17 days then a leveling of production to the end of the fermentation. The level of cyclosporins produced was not high, only 30 mg/L but the relative increase in cyclosporin levels was quite rapid initially, 2.4 mg/L/day. This indicated the fermentation time might be shortened considerably. An interesting feature of this experiment was that analysis of the final homogenate revealed the actual cyclosporin level was 90 mg/L which was much higher than the 27 mg/L the day 59 sample showed. This demonstrated a problem in sampling the fermentation which was also seen with the 2L fermenter (discussed later). The fact that most of the cyclosporin was mycelium-associated and the samples contained proportionally low biomass resulted in reduced cyclosporin levels in the samples. The fermentation produced reasonably high levels of cyclosporin despite a suboptimal medium and a very small inoculum (1%). Additionally, the casamino acids and sorbose were autoclaved separately which had been shown to yield lower levels of cyclosporin than autoclaving together.

Figure 15 Cyclosporin Production and pH of *T. niveum* UAMH 4594 over Time in a 10L Stirred Tank Fermenter

Precultures were started by inoculating 1.5 mL of a spore suspension washed from a sporulating plate of *T. niveum* UAMH 4594 into each of three flasks. These precultures were incubated 10 days then pooled. One hundred mL of preculture was used to inoculate a 10 L Microferm™ fermenter. The fermenter contained 9L of 1.6% sorbose medium and 1 L of 10% casamino acids which was autoclaved separately then added to give a total volume of 10L. After inoculation, the fermenter was run at 200 r.p.m. and an aeration of 3L/min. Samples (20 mL) were periodically taken via a sample port and the pH and cyclosporin content were determined. Loss in volume due to evaporation and sampling was replaced with distilled water. (Note that the ethyl acetate extracts were made up to 10 mL so cyclosporin levels are slightly decreased).


Cyclosporin Production (mg/L)
0
5
10
15
20
25
30
35
40
45
50
pH
4.0
4.5
5.0
5.5
6.0
6.5
Time (days)
0
5
10
15
20
25
30
35
40
45
50
55
60
Cyclosporin Production
pH

The fungus remained in a pelletized morphology throughout the fermentation due to the small inoculum and high shear forces in the fermenter which also accounted for the absence of growth on the fermenter walls. The mycelial dry weight of the fermentation was low, 2.2 g/L, but this was likely due in part to the small inoculum and the poor nitrogen source (casamino acids). An orange-brown pigment was produced which was carried through the ethyl acetate extraction but did not pass through the HPLC guard column frits, indicating that the pigment was particulate. Nearly 750 mg of crude cyclosporin was collected from two extractions of the fermentation concentrate (a 5:1 concentration by evaporating water).

It appears that the 10L stirred tank fermenter may be viable for cyclosporin production. The fact that a reasonably high level of cyclosporin was produced in an non-optimized system is promising. By using a superior carbon and nitrogen source, the production in the 10L fermenter might be increased to commercially useful levels.

3.9.2 Draught-Tube Airlift Fermenter (2 L)

The 2L LH™ airlift fermenter was obtained ten months into the project and immediately *T. niveum* fermentations were carried out. The fermenter design was new to this laboratory as fungal fermentations using external-loop airlift and stirred-tank fermenter designs had been used previously. A number of problems presented themselves and had to be solved before comparative studies on cyclosporin production between shake flasks and fermenters could be initiated. *T. niveum* UAMH 4594 was used initially, but UAMH 2472 was used after the first experiments.

The initial experiments carried out in the fermenter quickly became contaminated with another hyphomycete (not identified) which grew so quickly on the sorbose medium that the downcomer channel became plugged with mycelium. This problem was occasionally encountered in later runs. The chief accomplishment of the first fermenter runs was finally obtaining a fermenter which was run 792 h (33 days) with no contamination. The cyclosporin levels determined in this particular fermenter were never high, the peak production was observed in a sample taken at 6 days which contained 20 mg/L. The cyclosporin production declined to zero in the samples by 33 days. However when the contents of the fermenter were homogenized, cyclosporin content was found to be 57 mg/L (specific production was 26 mg/g). The discrepancy between the sample values and the final homogenate was puzzling and lead to an examination of the sampling procedure

The first rigorous attempt to produce cyclosporin in the 2L fermenter was then carried out with a majority of the possible sources of error identified and standardized. A 2% inoculum from a 7-day-old preculture (*T. niveum* UAMH 2472) was inoculated into the fermenter. Samples were withdrawn through the sample port, internal diameter=3.0 mm, after placing positive pressure on the vessel. The results showed there was a slow increase in cyclosporin levels to 46 mg/L in 28 days and then a rapid decline to 10 mg/L at 46 days. The final homogenate had a cyclosporin content of 110 mg/L of which a surprising quantity was found in the culture fluid (up to 33% of the total cyclosporin). This difference between the samples and final homogenate indicated the sample data was not an accurate reflection of the fermentation. The pH of the culture in this experiment began at 5.4 and after a short lag of 3 days increased in a linear fashion to 6.2. Whether this pH increase had any effect on the culture is unknown though it is interesting that pH in shake flask cultures remains quite constant throughout a fermentation (see 3.7.1).

As the sampling method had proved to be inadequate a different approach was undertaken. A suspension of 10^5 spores from the glycerol stocks was inoculated into the fermenter followed 12 h later by an additional 10^5 spores. The spores attached to the walls of the fermenter completely covering the bottom third of the outer walls. This mycelia continued to grow for 58 days at which time the mycelia was up to 1 cm thick on the walls (aeration had been increased after attachment to circulate the medium). The mycelium had a cyclosporin content of 8.8 mg/g (wet mycelium) and this cyclosporin was exclusively cyclosporin A (the most hydrophobic class ordinarily produced). The liquid portion of the culture had 17.3 mg/L cyclosporin. Only 20-30% of the total mycelium was extracted. One can see from these results that cyclosporin production from this type of "fluidized-bed" system was not significantly higher than any of the previous fermenter runs and was lower than shake flask cultures. The fluidized-bed approach did not show much promise and was not repeated.

Experiments were carried out with a modified central draught-tube fermenter design. The modification was suspending the draught-tube from the top plate of the fermenter. Cyclosporin was produced in only trace amounts (<5 mg/L) and the modification to the fermenter had little effect on reducing mycelial attachment. The only significant feature of these fermentations was the large amount of foam produced but analysis of the foam for cyclosporins was not carried out. Additional experiments with the fermenter using differing inocula failed to yield high levels of cyclosporin in either the samples or final homogenates. The use of alginate beads in the fermenter allowed good circulation and little adhesion to the fermenter walls but the beads soon broke apart as the alginate dissolved in the medium which had no added Ca^{++} to stabilize the alginate (see 3.4.3)

The biggest problem with these experiments was accurate sampling. A sample port was built into the top-plate of the LH™ fermenter so naturally this was used for sampling but the size of many of the mycelial clumps and pellets exceeded the diameter of the sample tube. The resulting sieveing effect restricted the samples to small bits of mycelia and culture fluid. This type of sample was not representative of the whole culture in the fermenter and sample data was viewed with this limitation in mind.

From these experiments with the 2L airlift fermenter, several conclusions can be drawn. Firstly, the design allows a large amount of hyphal attachment to the vessel's internal surfaces. This attachment was reduced though not eliminated by silanizing the vessel. Secondly, while in several experiments high cyclosporin levels were found in the culture homogenate, the samples were not always correspondingly high and cyclosporin production was never as high as shake flask cultures. These problems must be rectified in order to carry out studies of cyclosporin production over time as was done for shake flask cultures (see 3.9.4 for further experiments comparing production in the 2L fermenter and the 1L external loop fermenter).

3.9.3 External Loop Airlift Fermenter (1 L)

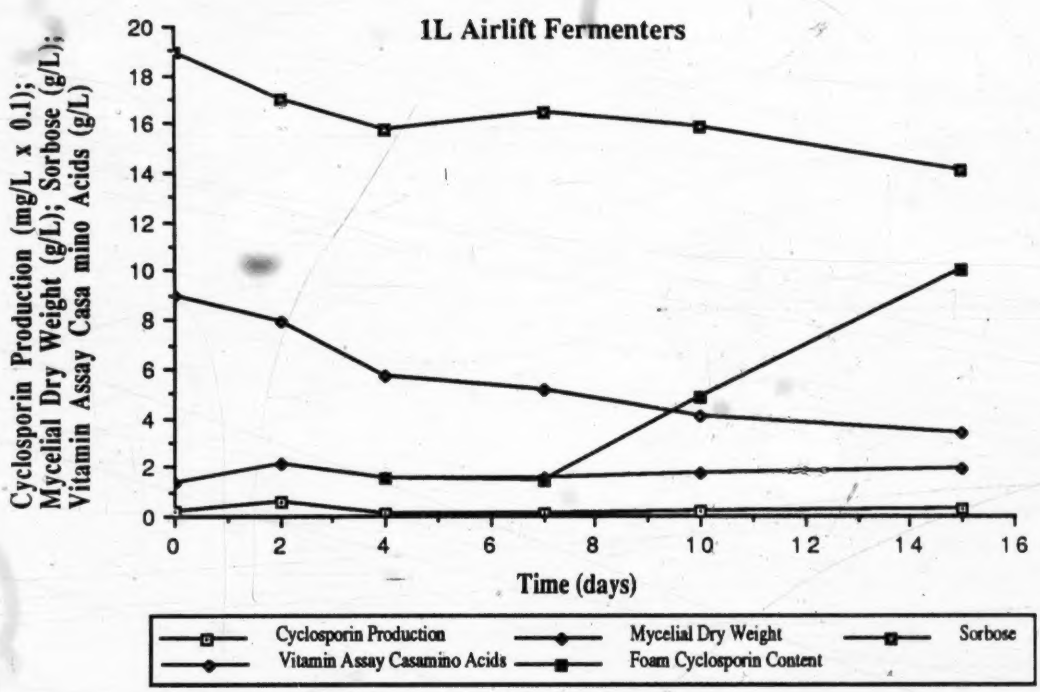
The 1L external loop fermenter is a design which has been frequently used in this laboratory for the production of chloroperoxidase (Carmichael and Pickard, 1986). The design had proved useful for the growth of both entrapped mycelia and fungal pellets. Five fermenters of this type were available for comparative studies on cyclosporin production by *T. niveum*. There were no sampling ports in these fermenters as was the case with the LH™ 2L fermenter or the Microferm™ 10L fermenter. Therefore the cyclosporin levels recorded were those of the culture homogenate. The number of experiments carried out with this design was limited by availability of the fermenters.

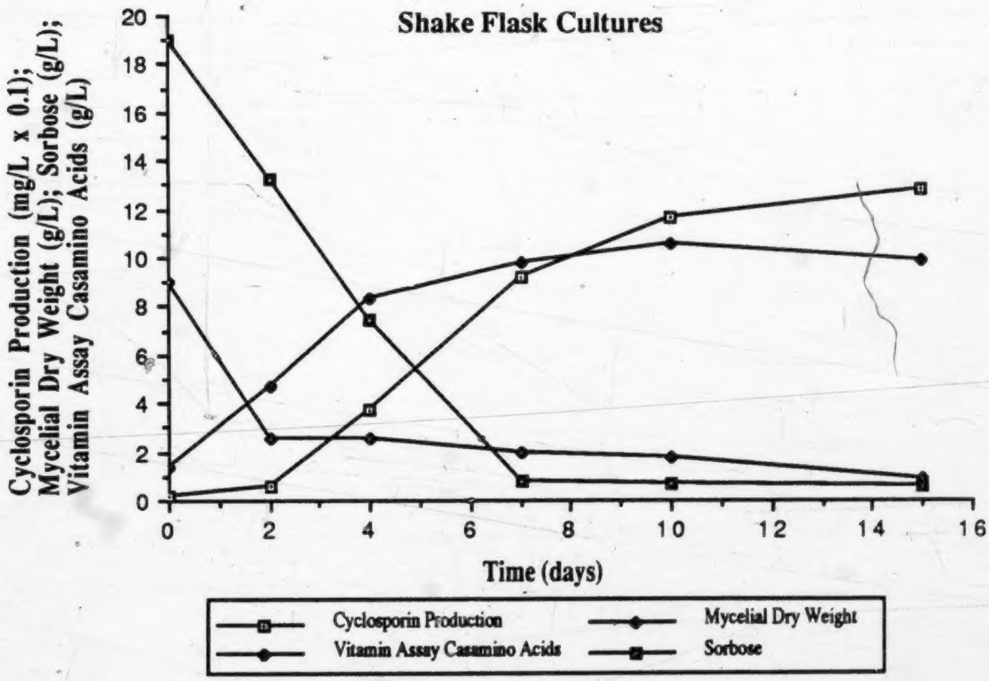
The first experiment was carried out using 1.6% sorbose medium-1% casamino acids and *T. niveum* UAMH 4594. The fermenter received a 2.5% inoculum (25 mL) from a 72 h preculture which had been started from spores washed from a sporulating plate. Airflow was kept to a minimum during the experiment. The fermenter was run for 31 days then stopped and the contents homogenized and analyzed. The cyclosporin content of this final homogenate was 55 mg/L (specific production was 32 mg/g dry weight). Although the production of cyclosporin was not as high was in shake flask cultures, the specific production was the highest specific production observed. However part of this high value might have been due to the long time period of the fermentation (31 days). This experiment showed that cyclosporin could be produced readily in the 1L fermenter and at high specific production levels. The natural development would be to increase the mycelial growth in the fermenter. This might prove to be a problem as a large percentage of the inoculum was blown out of the medium by air bubbles but by limiting aeration to minimum this problem might be avoided. Several other experiments were carried out with this fermenter design (see 3.9.4).

After continued work with these fermenters, an experiment was carried out to follow the production of cyclosporin in the airlift fermenter and compare that production to shake flask cultures. Five similar 1L airlift fermenters and 11 flasks were inoculated at 10% with a 96 h. preculture of *T. niveum* UAMH 2472. The fermenters were all aerated at the minimum necessary for circulation and the flasks were agitated at 200 rpm. At 2, 4, 7, 10, and 15 days one fermenter and two flask cultures were harvested and analyzed for cyclosporin content, pH, mycelial dry weight, sorbose content, and vitamin assay casamino acids content. Additionally the foam from each fermenter was analyzed separately for cyclosporin content. The results (Figure 16) for the shake flasks closely followed those in Figure 11 where the sorbose and vitamin assay casamino acids were rapidly taken up by the fungus and cyclosporin rapidly increased to a maximum of 128 mg/L on day 15 (Figure 16). In contrast the fermenter yielded almost no cyclosporin from the extraction of the mycelial homogenate. However extraction of the foam from each fermenter showed increasing quantities of cyclosporin up to 101 mg by day 15 (foam included the mycelium which had been carried from the medium by the rising air bubbles). While this was lower than the production observed in the shake flasks this cyclosporin was much easier to recover, the foam could be skimmed off the top of the medium and extracted rather than homogenizing and extracting the whole culture. That such a large quantity of cyclosporin was produced by the fermenter cultures was surprising as the sorbose and

Figure 16 Comparison of *T. niveum* Growth and Cyclosporin Production in Shake Flask Culture and in a 1L Airlift Fermenter

Eight flasks containing 100 mL of SVA medium were each inoculated with 10^8 spores of *T. niveum* UAMH 2472 from glycerol stocks, incubated 96 h and pooled. Five 1L external loop airlift fermenters containing 1L of SVA medium were each inoculated with 100 mL of preculture. These fermenters were all incubated at 22°C with a minimum of aeration. Eleven flasks each containing 100 mL of the sorbose medium were inoculated with 10 mL of preculture and incubated at 27°C. One culture was harvested at time=0 h and used as start point for both fermenter and shake flask curves. At days 2, 4, 7, 11, and 15 one fermenter and two shake cultures were harvested. For the last four fermenters analyzed, the cyclosporin content of the foam and mycelium blown out of the medium was determined. The fermenter results are shown in the top graph and the shake flask results in the bottom graph.


1L Airlift Fermenters
Cyclosporin Production (mg/L x 0.1); Mycelial Dry Weight (g/L); Sorbose (g/L); Vitamin Assay Casa mino Acids (g/L)
0
2
4
6
8
10
12
14
16
18
20
Time (days)
0
2
4
6
8
10
12
14
16
Cyclosporin Production
Mycelial Dry Weight
Sorbose
Vitamin Assay Casamino Acids
Foam Cyclosporin Content


Shake Flask Cultures
Cyclosporin Production (mg/L x 0.1); Mycelial Dry Weight (g/L); Sorbose (g/L); Vitamin Assay Casamino Acids (g/L)
0
2
4
6
8
10
12
14
16
18
20
Time (days)
0
2
4
6
8
10
12
14
16
Cyclosporin Production
Mycelial Dry Weight
Vitamin Assay Casamino Acids
Sorbose

vitamin assay casamino acids were not taken up as quickly as in the shake flask cultures. This reduced uptake of nutrients may be indicative of reduced metabolism in the fermenters.

In conclusion it can be seen that the 1L airlift fermenter allowed considerable cyclosporin production in spite of low mycelial growth. The discovery of the high cyclosporin content of the foam portion of the culture places conclusions drawn earlier regarding fermenter production in question. The problems with sampling may have been reflective of the fact that most of the cyclosporin was in the foam and could not be sampled from a sample port 5 cm under the surface of the medium. This must be investigated further with the 2L as well as the 1L airlift fermenters. The objective for further experiments with the 1L airlift must be to increase mycelial growth in the fermenter and to reduce aggregation of the mycelia within the fermenter. The addition of junlon or hostacerin which have increased filamentous growth in other fungi (Jones *et al.* 1988) may facilitate these objectives.

3.9.4 Comparison of Fermenter Designs

The chief objective of the following experiments was to compare *T. niveum* growth and cyclosporin production in the 1L and 2L fermenters in parallel experiments with that in shake flask cultures. Precultures of *T. niveum* UAMH 2472 which were started with 10^8 spores and incubated 96 h were used to inoculate three 1L airlift fermenters, three shake flasks, and the 2L airlift fermenter containing 2% sorbose medium-1% vitamin assay casamino acids using a 3% inoculum. The shake flask cultures were incubated 15 days, the 2L airlift was aerated 8 days before the downcomer became plugged with mycelium, and the 1L airlift fermenters were aerated for 21 days. The cyclosporin production of these cultures is given in Table 16.

The uptake of sorbose and vitamin assay casamino acids was not determined for

Table 16 Cyclosporin Production in Fermenters and Shake Flasks

	Mycelial Dry Weight (g/L)	Cyclosporin Prod. (mg/L)	Specific Cyclosporin Prod. (mg/g)
Shake Flasks	8.5	164	19
2L airlift	3.2	9	3
1L airlift	5.4	105	16

Precultures were started with 10^8 spores of *T. niveum* UAMH 2472 in 100 mL of SVA medium. These precultures were incubated 96 h. 3% inoculations were made into triplicate shake flasks, triplicate 1L fermenters, and one 2L fermenter all containing SVA medium. The fermenters were aerated such that the mycelia was just circulating whille the shake flasks were agitated at 200 rpm. These production flasks and fermenters were incubated for 15 days and the shake flasks were made up to volume with distilled water. The cultures were homogenized and analyzed.

any of the cultures. The higher cyclosporin levels observed in the 1L fermenter may be due to the increased fermentation time of 21 days (compared to 15 days in Figure 16). This type of experiment should be repeated to confirm the findings here and to investigate the relationship between nutrient uptake and progress of the fermentation (compared to time course data). It might show that sorbose content or mycelial dry weight may be indicative of the progress of cyclosporin production.

Another experiment was carried out comparing the two designs of airlift fermenter but with a different perspective. Rather than inoculating precultures which were growing as filamentous mycelia, pellets from precultures were inoculated. These pellets were incubated 15 days then the pellets were transferred to shake flasks for 5 days while the fermenters were cleaned, silanized, and sterilized. At this point the beads were again weighed and replaced into the fermenters. The fermenters were then run an additional 42 days and harvested. The results of this experiment are not be presented because the pellets did not contain any cyclosporin. Analysis for cyclosporins was negative though a number of other compounds which might have been breakdown products of cyclosporin were observed. The lack of cyclosporin may be related to the length of the fermentation or the morphology of the fungus. The pellets which were finally harvested were large in diameter (1 cm) but were hollow, the centre of the pellets had lysed, likely due to oxygen deprivation. Only the outer edge of the pellets (1-2 mm thick) was actually mycelium. The wet weights of the pellets increased throughout the experiment but whether the mycelial weight, dry mycelial weight increased accordingly was unknown. These pellets were 8% mycelium by weight, the remainder was water. Therefore one can conclude that under the conditions used, pellets were not useful for the production of cyclosporin and the only cyclosporin produced was from the mycelia sheared off the pellets in the shake flasks between fermenters.

The conclusions which can be drawn from these experiments are quite straightforward. The 1L airlift fermenter design was superior to the 2L design for both mycelial growth and cyclosporin production. In most experiments the 1L production values were just slightly less than the shake flask control values. The 1L design was convenient to use and presented fewer problems than the 2L design in term of sterilization, inoculation, and mycelial adhesion. In the future, experiments with these fermenters should focus on increasing mycelial growth and enhancing filamentous growth.

Chapter 4: Discussion

Cyclosporin fermentations have not been well described in the literature: developments in the microbiological aspect of production have been few. From the initial report of cyclosporin discovery by Dreyfuss *et al.*(1976) to the start of this project in fall of 1985, only five reports had been published describing the methodology involved in producing cyclosporins. These five papers, three from workers at Sandoz Ltd., describe a number of media and strains of *T. inflatum* which were mutants derived from the parent NRRL 8044 strain. Only Foster *et al.* (1983) used the parent NRRL strain and high cyclosporin production was achieved, 200-300 mg/L, but no other results have been published since their 1983 paper. Cyclosporin production in all these papers was in the range of 150-300 mg/L for control cultures and up to 750 mg/L in directed synthesis experiments but no indication of reproducibility of these values was given. Therefore a project to optimize the production of cyclosporin must start essentially from the data of Foster *et al.* (1983) as this work used the parent *T. inflatum* NRRL strain and not the mutant strain of the Sandoz workers.

In the two years since the start of this project only two papers dealing with the microbiological aspects of cyclosporin production have been published, both from the group led by S. Agathos (Agathos *et al.* 1986 and 1987). These two papers deal primarily with screening a large number of carbon and nitrogen sources for cyclosporin production. The findings which have been published are a reflection of the work they have been allowed to publish subject to the approval of their funding source (personal communication to M.A. Pickard). However beyond this no other microbiological papers have been published on cyclosporin production by *Tolypocladium sp.* This presents the problem of having no external guide as to methodologies which have worked for other researchers so all development must come from within our lab.

Development of methodology must start at some point so a number of assay procedures were looked at for both detecting and quantifying cyclosporin levels. A bioassay which used inhibition of sporulation of *A. niger* by cyclosporin as the indicator was examined. It was found however that this method, which was certainly qualitatively indicative of cyclosporin activity, was not even semi-quantitative. Problems were also found with the assay relating to handling large numbers of *A. niger* spores. Other assay systems which had been used for cyclosporin quantitation included an RIA and HPLC analysis. The RIA was commercially available and is specific for cyclosporin A which is the only therapeutically used class of cyclosporin. The RIA had also been found to overestimate the quantities of cyclosporin compared to HPLC analysis likely as the assay was also cross reactive for metabolites of cyclosporin A (Carruthers et al. 1983). The final method, HPLC analysis, had few drawbacks other than access to an HPLC. For the first eight months of this project this access was limited to part-time use until an HPLC was purchased for cyclosporin determinations. Therefore experiments were often limited until the results of preceding experiments were obtained.

The initial experiments carried out were found to have a large degree of variability both between the strains being tested and duplicate flasks of these strains. Despite this variability it was obvious that the NRRL strain of *T. inflatum* was one of the poorer cyclosporin-producing strains. Therefore a comparison of all available strains if *Tolypocladium* sp. was made. Five strains of *T. niveum* were found to produce cyclosporin in significantly higher quantities than five other strains of *T. niveum* and the *T. inflatum* strain. One of these five strains, UAMH 2472, was then used to optimize the methodology involved in producing cyclosporin.

The first difficulty arising was the isolation of cyclosporins from the culture. Extraction with an organic solvent was the procedure previously followed in the literature, but a number of solvents had been used including butyl acetate, ethyl acetate, and methanol. A comparison between these showed ethyl acetate the best solvent for this purpose. Quantification of cyclosporin in the ethyl acetate was carried out by HPLC analysis and proved to be both reproducible and efficient though quite time-intensive. Each analysis required 10-15 minutes to complete and was done in duplicate therefore a single sample required up to 45 min for analysis. The inoculum for production has been optimized from a preculture inoculum of 10^8 spores/100 mL SVA medium and a 96 h incubation time before a 10% inoculum of preculture into the production flasks. The SVA medium has also been optimized for cyclosporin production. The results of many experiments show that SVA medium (2% sorbose , 1% vitamin assay casamino acids, 1% KH_2PO_4, and 0.5% KCl) was best for cyclosporin production. This medium is much simpler than the media used previously in the literature. Once these problems had been overcome by optimizing conditions for cyclosporin production, the nine strains of *T. niveum* should be screened again to find the highest producers of cyclosporin and the growth curve experiments repeated if new strains of *T. niveum* show themselves as the highest producers of cyclosporin.

A number of conclusions can be drawn from this research. The most important is that cyclosporin can now be produced from several strains of *T. niveum* in high and reproducible quantities. The strain most often used in the literature, *T. inflatum* NRRL 8044 still produced only small quantities of cyclosporin even after the conditions had been optimized for cyclosporin production. The medium which resulted from optimization for cyclosporin is much simpler than that used by Kobel and Traber (1982) which included sucrose, malic acid, ammonium, vitamins, and trace metals.

Several carbon sources have been shown to support *T. niveum* growth and cyclosporin production including sorbose, fructose, and to a lesser extent glucose. It appears that the complex nitrogen source is more important for the production of cyclosporin than the carbon source. Vitamin assay casamino acids have been show to be the best nitrogen source available though high cyclosporin yields were obtained in the initial experiments using casamino acids. Later experiments with casamino acids yielded only low cyclosporin production. This discrepancy cannot be readily explained and as the bottle and lot number were not recorded at that time, the explanation will likely not be forthcoming. The reason for the higher cyclosporin production on vitamin assay casamino acids than on casamino acids is at present unknown. The biggest differences between casamino acids and vitamin assay casamino acids, besides the obvious fact that there are no vitamins in vitamin assay casamino acids, are the salt content and the higher content of aspartic acid, glutamic acid, proline, serine, and alanine in vitamin assay casamino acids. In addition vitamin assay casamino acids have been shown to contain oleate and sterate which are growth factors in some organisms (Nolan, 1971). The casamino acids have 10% total nitrogen, 14% NaCl, and 20% ash with only 5% glutamic acid and 0.5 % aspartic acid of the total amino acids while the vitamin assay casamino acids have 7% total nitrogen, 38% NaCl, 41 % ash with 15% glutamic acid and 5% aspartic acid (Nolan & Nolan, 1972). Whether these differences are the reason for the improved cyclosporin yields is unclear, further experiments involving supplementation of casamino acids with aspartic acid, glutamic acid, and proline will be carried out to determine the reason for the superior production on vitamin assay casamino acids.

Production of cyclosporin in several designs of bench top fermenters was carried out but cyclosporin production levels varied both between fermenter designs and duplicate runs. However the 1L external loop airlift fermenter appears to show the most promise for further experimentation. The design is the simplest of the three used and yielded the most consistent results. The stirred tank fermenter and 2L draught-tube fermenter did not show much promise due to lack of availability of the former and inconsistent results from the latter. As optimization of cyclosporin production by altering the medium continues these developments should be used to make fermenter production of cyclosporin a more viable undertaking.

The procedure in extracting the cyclosporin involved extracting a sample of whole culture, mycelia and liquid, to ensure all the cyclosporin in the sample was accounted for. In related work it was found that the ratio of cell-associated cyclosporin to soluble cyclosporin varied over time. From day 1-10 of a fermentation the cyclosporin was over 90% cell-associated. However after this time an increasing percentage was found in the liquid phase of the culture. In an extreme instance, a 2L fermenter had 30% of its total cyclosporin in the liquid phase of the culture after thirty days. The increasing quantities of cyclosporin found in the liquid phase may be due to breakdown of the mycelium after active growth has stopped. If the cyclosporin is intracellular or cell-associated then lysis of the mycelium would make the cyclosporin more available for dissolution in the medium. That such a high percentage would dissolve in the medium is unexpected due to the hydrophobic nature of the molecule and its insolubility in water alone. This hydrophobic nature might be exploited in fermenter production where in one instance the foam and mycelia which collected at the top of the 1L fermenter had a very high cyclosporin content (100 mg of cyclosporin in the foam) while there was virtually no cyclosporin found in the culture, either cell-associated or soluble. Because of the uncertainty in the location of the cyclosporin, the whole culture was routinely extracted.

Cyclosporin production has been reported as total cyclosporin in the culture. However this value is the sum of the three ordinarily observed classes of cyclosporin. The ratio of these classes is typically 66:10:24 for cyclosporins A:B:C though slight variations may occur. In general the 65-70% of the total cyclosporin reported can be considered cyclosporin A. The elution times of the cyclosporins varied as the column aged, elution times shortened until resolution of peaks was no longer clear at which point this column was replaced. The elution time for cyclosporin A, the last class to be eluted from the reverse phase column was typically from 7-10 minutes. Therefore only 3-4 samples could be analyzed per hour as there was an interval required for the detector to return to baseline. Separation of the three classes of cyclosporins was good, peak resolution never became a problem. If the peak areas varied between duplicate injections by more than 5-10% repeated injections were made until the peak areas were both reproducible and in agreement. As described the bioassay was useful only as an indicator of cyclosporin activity, further work would be necessary to develop a quantitative assay from this activity.

The methodology involved for extracting and quantifying cyclosporin production has been rigorously examined. The values obtained from the extraction and HPLC analysis have been shown to be representative of the cyclosporin content of the culture. However there is inherent variability in cyclosporin production between replicate flasks. This variability led to the use of multiple flasks for each experiment. Until the variability had been reduced each experiment was carried out using quintuplicate flasks, limiting the number of experiments which could be carried out due to the availability of equipment and the length of analysis of each sample. Once there was good agreement between replicate samples, and to expedite the research, the number of replicate flasks was reduced to three. There appears to have been no problem using triplicate flasks but there is inherently less confidence in the values obtained. The variability between replicate flasks made analysis of the results difficult as in many cases the range of two groups of flasks would overlap. To aid in analysis, Duncan's Multiple Range test was used on the data to find statistical differences between groups of data. This proved valuable in determining if a certain treatment improved cyclosporin production compared to the control and if the differences were significant.

In conclusion it should be stated that the rationale for this study was to elucidate the cyclosporin production of *T. niveum* which have not been described as producers. This production could not be properly determined until methods for the detection of cyclosporin had been found and possibly improved. The literature provided little assistance in this matter. In many cases descriptions of methodologies lacked the detail necessary for duplication of these experiments. Considering the economic factors involved in cyclosporin production this reluctance to release details of its production is understandable. Therefore the majority of methods used in this study, if not developed from scratch, had to be thoroughly tested to determine their validity before they could be confidently used.

There are a great number of avenues future studies could follow. Continued studies into optimization of the growth medium, especially with regard to the nitrogen source. The supplementation of casamino acids with aspartic acid and/or glutamic acid and/or proline may result in an increase in cyclosporin production. The reasons for the difference in cyclosporin production between casamino acids and vitamin assay casamino acids should be resolved. Possibilities for this difference range from the increased salt concentration to even the higher ash content. Some fungi have been shown to require the ash component of these casein hydrolysates for growth (Nolan and Nolan, 1972). Though several carbon sources have been shown to yield high levels of cyclosporin, the rapid uptake of sorbose and the concurrent production of cyclosporin with increasing mycelial weight suggests that a two-stage feeding of the culture might result in higher final cyclosporin production. This had been observed by Agathos *et al.* (1986 and 1987) in fed-batch cultures using sorbose then adding maltose to yield higher cyclosporin production levels. This two-stage feeding might include addition of carbon source or nitrogen source to growing cultures. Supplementation of the medium with constituent amino acids has been shown to direct synthesis of cyclosporins as well as increase the final production (eg. L-valine).

This might be pursued further with the supplementation to include several amino acids added to a culture. The results of the two strategies of medium supplementiation suggest that cyclosporin production can be increased and/or directed by adding substituent amino acids. The addition of an amino acid such as L-valine both increased cyclosporin production and directed synthesis to cyclosporin A. This is the desired effect of supplementation. Whether this supplementation is commercially viable or can be repeated requires further investigation as do the effects of adding several amino acid to the cultures or acetate to expedite formation of the amino acid L-C_9 should be investigated. However supplementation with methyl donors has yielded no increase in cyclosporin production. Cyclosporin might also be increased by removing certain components of the medium.

Future studies might also deal with fermenter production of cyclosporin. Work with the 1L airlift fermenter has been promising both in quantity of cyclosporin produced as well as ease in recovering the product. The slow uptake of sorbose and vitamin assay casamino acids presents the question of whether the fermenter cultures would take up these nutrients over a longer period of time and if cyclosporin levels would concurrently increase. Also the low mycelial growth in the fermenter might be increased by a larger inoculum which might also increase the rate of sorbose and vitamin assay casamino acids uptake. These questions remain to be investigated. Fermenter production of cyclosporin should not be the main thrust of research until the other areas, including medium optimization have been completed.

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